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Inflammatory Biomarkers in Multiple Sclerosis

ACADEMIC DISSERTATION
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To my family

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LIST OF ORIGINAL PUBLICATIONS

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I. Rinta S, Kuusisto H, Raunio M, Paalavuo R, Levula M, Lehtimaki T, Elovaara I. Apoptosis-related molecules in blood. Journal of Neuroimmunology 2008; 205: 135-141.

II. Hagman S, Raunio M, Rossi M, Dastidar P, Elovaara I. Disease-Associated Inflammatory Biomarker Profiles in Blood in Different Subtypes of Multiple Sclerosis: Prospective Clinical and MRI Follow-Up Study. Journal of Neuroimmunoly 2011; 234: 141-147.

III. Hagman S, Raunio M, Lehtimäki T, Vihinen M, Kähönen M, Dastidar P, Elovaara I. Aberrant expression of apoptosis related genes in MS: a new prognostic marker? (submitted)

IV. Rinta S, Airas L, Elovaara I. Is the modulatory effect of pregnancy in multiple sclerosis associated with changes in blood apoptotic molecules? Acta Neurologica Scandinavica 2010; 122: 168-174.

ABBREVIATIONS

APAF	Adaptor apoptotic protease activating factor
BAD	BCL2-associated agonist of cell death;
BBB	Blood-brain barrier
BBC3	BCL2 binding component 3;
BCL-2	B-cell lymphoma 2
BCL2L14	Apoptosis facilitator Bcl-2-like protein 14
BIRC	Baculoviral IAP repeat-containing
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
CDMS	Clinically definite MS
CFLAR	CASP8 and FADD-like apoptosis regulator
CIS	Clinically isolated syndrome
CCL	C-C motif chemokine ligand
CNS	Central nervous system
CSF	Cerebrospinal fluid
CXCL	C-X-C motif chemokine ligand
DC	Dendritic cells
DMT	Disease-modifying therapy
DR	Death receptors
EDSS	Expanded disability status scale
EAE	Experimental autoimmune encephalomyelitis
FasL	Fas ligand
FLAIR	Fluid attenuated inversion recovery
GA	Glatiramer acetate
Gd	Gadolinium
HLA	Human leukocyte antigen
IFN	Interferon
IKBKE	inhibitor of nuclear factor kappa-B kinase subunit epsilon

IL	Interleukin
MBP	Myelin basic protein
MIF	Macrophage migration inhibitory factor
MMP	Matrix metalloproteinase
MS	Multiple sclerosis
MOG	Myelin oligodendrocytes glycoprotein
MRI	Magnetic resonance imaging
MP	Methylprednisolon
NAWM	Normal-appearing white matter
NF	Neurofilament
NF-κB	Nuclear factor kappa B
NFKBID	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
NK	Natural killer cell
NO	Nitric oxide
RRMS	Relapsing-remitting MS
OCB	Oligoclonal bands
PBMC	Peripheral blood mononuclears cells
PPMS	Primary progressive MS
S	Soluble
SPMS	Secondary progressive MS
TCR	T-cell receptor
Th	CD4+ T helper cells
TNF	Tumor necrosis factor
TNFRSF25	Tumor necrosis factor receptor superfamily, member 25
TRAIL	TNF-related apoptosis inducing ligand
Treg	Regulatory T cell

ABSTRACT

Multiple Sclerosis (MS) is an immune-mediated disease of the central nervous system (CNS) that is characterized by a variable clinical course and heterogeneous and complex pathology. Pathophysiological processes in MS contribute to the disease course and clinical manifestations, and therefore biomarkers that are indicative of these events would provide significant potential for diagnostics, prediction of disease course and optimization of therapeutic responses.

The goal of this thesis was to identify biomarkers in the blood that could reflect pathogenetic processes in MS and be used as biomarkers of disease activity and progression. In this regard, the aims were to: 1) define immune profiles in different clinical subtypes of MS and clinically isolated syndrome (CIS); 2) search for biomarkers for disease activity and disability; 3) search for prognostic biomarkers for CIS to MS, and 4) explore whether the postpartum disease activation of MS is related to changes in the apoptotic molecules in the blood. The study included patients with different subtypes of MS, CIS and healthy controls that underwent neurological, magnetic resonance imaging (MRI) and immunological examinations.

Altered expression of immune profiles in MS subtypes were found on both the protein and gene levels. Immune profiles in sera of primary progressive MS (PPMS) were characterized by elevated levels of tumor necrosis factor (TNF)-α, soluble Fas (sFas) and C-C chemokine motif ligand 2 (CCL2). Decreased serum levels of macrophage migration inhibitory factor (MIF) were found in relapsing-remitting MS (RRMS). Aberrant expression of six apoptosis-related genes (*BAD, BBC3, BCL2L14, TNFRSF25, IKBKE, NFKBID*), including B-cell lymphoma 2 (Bcl-2) and nuclear factor kappa B (NF-κB) families and death receptor pathway, were found in RRMS patients. Interestingly, atypical expression profiles of apoptosis-related genes were also seen in CIS (*BNIP3, TNFRSF25, IKBKE*).

Active disease course was associated with upregulation of serum MIF and TNF-related apoptosis inducing ligand (TRAIL), and disease progression was associated

with increased TRAIL mRNA, MIF and sTRAIL. In CIS, elevated expression of three apoptosis-related genes (*APAF, BIRC6, CFLAR)* was found in those patients who fulfilled the diagnostic criteria for MS over the two-year follow up.

In the study of the immunomodulatory effect of pregnancy on MS, sTRAIL and sFasL were upregulated in both MS patients and healthy women from late pregnancy to postpartum. The increase in sTRAIL was significantly smaller in the MS patients in comparison with the controls.

In summary, this thesis focusing on the identification of biomarkers in MS showed that PPMS was characterized by elevated levels of TNF-α, sFas and CCL2 indicating inflammatory activity in this subtype. Abnormal expression of apoptosis-related genes in RRMS and CIS suggested an enhanced potential for apoptosis in immune cells directed at controlling MS disease activity. Disease activity was associated with increased levels of serum TRAIL and MIF and disease progression was associated with upregulated levels of sFas, MIF and TRAIL mRNA, suggesting these molecules to be candidate biomarkers for disease activity and progression. Interestingly, three new prognostic biomarkers for conversion to MS were found. Increased MS disease activity postpartum may be related to inadequate inhibition of T-cell reactivation after pregnancy. Currently we are validating these findings using larger patient series and longer follow-up periods.

TIIVISTELMÄ

Multippeliskleroosi (MS) alkaa nuorella aikuisiällä ja johtaa vuosien kuluessa invaliditeetin kertymiseen. Sairauden syytä ei toistaiseksi tunneta, mutta sen kliinisessä kuvassa ja patogeneesissä esiintyy monimuotoisuutta ja yksilökohtaista vaihtelua. Tärkeänä tavoitteena MS-taudin hoidon kehittämisessä on tunnistaa sairauden immunologiset alatyypit, joka edistäisi yksilöllisen hoidon edellytyksiä ja täsmälääkkeiden käyttöönottoa.

Väitöskirjatyön päätavoitteena oli tunnistaa taudin patogeneesiä kuvaavia veren immunologisia profiileja MS-taudin eri alatyypeissä. Lisäksi tutkimuksen tavoitteena oli identifioida biomerkkiaineita, jotka kuvaavat taudin aktiivisuutta ja sen etenemistä ja jotka ennustaisivat MS-taudin kehittymisen riskiä, sekä selvittää onko MS-taudin aktivoituminen synnytyksen jälkeen yhteydessä muutoksiin apoptoottisten molekyylien ilmentymisessä. Tutkimus koostui MS-potilaita, kliinisesti eriytyneestä oireyhtymästä (KEO) ja terveistä kontrolleista, jotka tutkittiin neurologisesti ja aivojen kuvantamisella sekä heidän verinäytteistä tutkittiin ehdokasbiomerkkiaineiden ilmentymisprofiileja.

MS-taudissa poikkeavia immuniprofiileja ilmentyi sekä proteiini- että geenitasolla. Primaarisprogressivisessa MS-taudissa havaittiin kohonneita TNF-α, Fas ja CCL2 pitoisuuksia. Lisäksi havaitsimme Bcl-2 ja NF-κB perheiden ja kuolemareseptori signalointireitille kuuluvien kuuden geenin yli-ilmentyneen aaltomaisessa MS-taudissa. Myös KEO-potilailla havaittiin poikkeavuutta apoptoottisten geenien ilmentymisessä. Aaltomaisen MS-taudin aktiivisuus liittyi TRAIL ja MIF proteiinien pitoisuuksien lisääntymiseen. Invaliditeetin lisääntyminen oli puolestaan yhteydessä kohonneisiin TRAIL mRNA pitoisuuksiin, sekä kohonneisiin seerumin MIF ja Fas pitoisuuksiin. Lisäksi, kolmen geenin ilmentyminen havaittiin olevan koholla niillä KEO-potilailla, joilla MS-diagnoosi varmentui kahden vuoden seurannan aikana. Tutkimuksessa raskauden immunomoduloivasta vaikutuksesta MS-taudin aktiivisuuteen havaittiin seerumin

TRAIL ja FasL:n tasojen kasvavan raskauden aikaisesta synnytyksen jälkeiseen tilaan sekä MS-potilailla että terveillä naisilla. Seerumin TRAIL:n muutoksen havaittiin olevan pienempi MS-potilailla kuin terveillä.

Tämän väitöskirjatutkimuksen tavoitteena oli tunnistaa MS-taudin seurantaan soveltuvia biomerkkiaineita. Kohonneet seerumin TNF-α, Fas ja CCL2 pitoisuudet primäärisprogressiivisessa MS-taudissa kuvastivat meneillään olevaa tulehduksellista aktiivisuutta tässä tautityypissä. Apoptoottisten geenien poikkeavat ilmentymisprofiilit aaltomaisilla ja KEO potilailla viittaa lisääntyneisiin apoptoottisiin mekanismeihin taudin aktiivisuuden hillitsemiseksi. Uusia kandidaatti biomerkkiaineita havaittiin, joista kohonneet TRAIL ja MIF tasot saattavat olla yhteydessä aaltomaisen taudin aktiivisuuteen, kun taas kohonneet Fas ja MIF proteiinien tasot ja TRAIL mRNA:n ilmentyminen invaliditeetin kertymiseen. Lisäksi useita ennustavia kandidaattibiomerkkiaineita löydettiin, joiden yli-ilmentyminen näyttää olevan yhteydessä MS-taudin kehittymisen riskiin. Lisääntynyt MS-taudin aktiivisuus raskauden jälkeen selittyneen häiriöinä immuunijärjestelmän aktivaatiota säätelivissä mekanismeissa. Saatujen tuloksien varmentaminen edellyttää suurempaa potilasaineistoa sekä pidempää seuranta-aikaa.

INTRODUCTION

Multiple sclerosis (MS) is an immune-mediated demyelinating disease of the central nervous system (CNS), where the pathological hallmarks are demyelination, axonal loss and inflammation (Trapp and Nave, 2008; Lassmann, 2009; Stadelmann, 2011). MS is the major cause of the neurological disability in young adults and therefore it has substantial personal, social and economic costs. MS is highly prevalent in northern countries, particularly in Finland where the prevalence of MS is among the highest in the world (Compston, 1997; Sumelahti et al., 2001).

MS is characterized by a variable clinical course and heterogeneous and complex pathology and pathogenesis. There are three clinical subtypes of MS involving relapsing-remitting MS (RRMS), secondary progressive MS (SPMS) and primary progressive MS (PPMS). Most patients initially have RRMS, which is characterized by relapses followed by subsequent improvement. After 10 to 20 years of RRMS, a majority of these patients will enter a phase of progression with or without attacks, called SPMS. A minority of patients (15%) have PPMS from onset. These patients' disease progresses slowly without relapses (Lublin and Reingold, 1996; Compston and Coles, 2002). The underlying pathological mechanism involved in CNS destruction is also heterogeneous. Currently pathological studies have suggested four different immunopathological patterns in demyelination in early MS lesions, which differ between patients. Although MS is considered to be as a white matter disease, grey matter is also affected (Lucchinetti et al., 2000; Lassmann et al., 2001; Lassmann et al., 2007).

MS disease is considered to be mediated by actions of inflammatory lymphocytes, which transmigrate into the CNS and initiate tissue damage and neurological impairment. Based on the current understanding, autoimmune-driven processes are believed to initiate the disease and the role of myelin specific CD4+ T helper (Th) cells type 1 and Th17 cells as driving force in the autoimmune processes, but also other cell types like, CD8+ T cells, B cells, macrophages and natural killer (NK)

cells contributes to the pathogenesis of MS (Sospedra and Martin, 2005; Kasper and Shoemaker, 2010). Programmed cell death, apoptosis of immune cells, is also an important pathophysiological mechanism in MS inflammation. It regulates elimination of autoreactive T and B cells and macrophages from the circulation and prevents their entry into the CNS. Dysregulation of apoptosis and other immune functions may lead to inflammation within CNS tissue and subsequent development of tissue damage (Zipp, 2000; Pender, 2007).

Several pathophysiological processes such as inflammation, demyelination and axonal damage mediate disease manifestation of MS. Since future therapeutic agents will probably be targeted specifically on pathophysiological pathways, the identification of possible immunological subtypes of individual MS patients could allow more focused application of different therapies (Lutterotti et al., 2007). Therefore, there is urgent need for an *in vivo* biomarker that would reflect underlying immunopathogenesis in MS patients. Moreover, the ultimate goal in the discovery of biomarkers in MS is to develop tools to predict disease outcome, to assess disease activity more accurately, and to predict therapeutic responses.

REVIEW OF THE LITERATURE

1. Overview of multiple sclerosis

1.1 Epidemiology of multiple sclerosis

The etiology of MS is still largely unknown, but there is evidence for a role of both genetics and environmental factors (Figure 1) (Ramagopalan et al., 2010). It is suspected that an infectious agent may trigger the disease in genetically susceptible individuals. The geographic distribution of MS is an epidemiological hallmark of it. In general, the prevalence of MS is lower in populations living near the equator and increases with the latitude in both the northern and southern hemispheres. The disease is most common in populations of northern European descent, especially in Scandinavia, the British Isles, the northern tier of the United States and southern Canada (Compston, 1997). In these regions the prevalence of MS is about 100/100 000. In certain parts of Finland the prevalence is 200/100 000 (Sumelahti et al., 2000; Sumelahti et al., 2001).

Genetic factors: The role of the genetic factors is supported by the studies of MS families. Concordance rates have been 20-30% in monozygotic twins versus 3-5% in dizygotic twins in these populations (Sadovnick et al., 1993). Siblings have a 15-40 fold increase risk of having MS and there is no effect of shared environment in adoptees and spouses (Ebers et al., 1995; Robertson et al., 1996; Sadovnick et al., 1996; Ebers et al., 2000). Among the genetic factors, the human leukocyte antigen (HLA) *DRB1*1501* allele exerts the strongest genetic effect on MS (Barcellos et al., 2006; Yeo et al., 2007). Genome-wide association studies have revealed the existence of multiple non-HLA genes with modest effect on MS, including interleukin-7 receptor α (*IL7RA*), interleukin-2 receptor α (*IL2RA*), C-type lectin-

domain family 16 member A (*CLEC16A*), lymphocyte function-associated antigen-3 (*LFA-3; CD58*), tumor-necrosis-factor receptor superfamily member 1A (*TNFRSF1A*), interferon regulatory factor 8 (*IRF8*) and *CD6* (International Multiple Sclerosis Genetics Consortium et al., 2007; Ramagopalan et al., 2007; International Multiple Sclerosis Genetics Consortium (IMSGC), 2008; Rubio et al., 2008; De Jager et al., 2009).

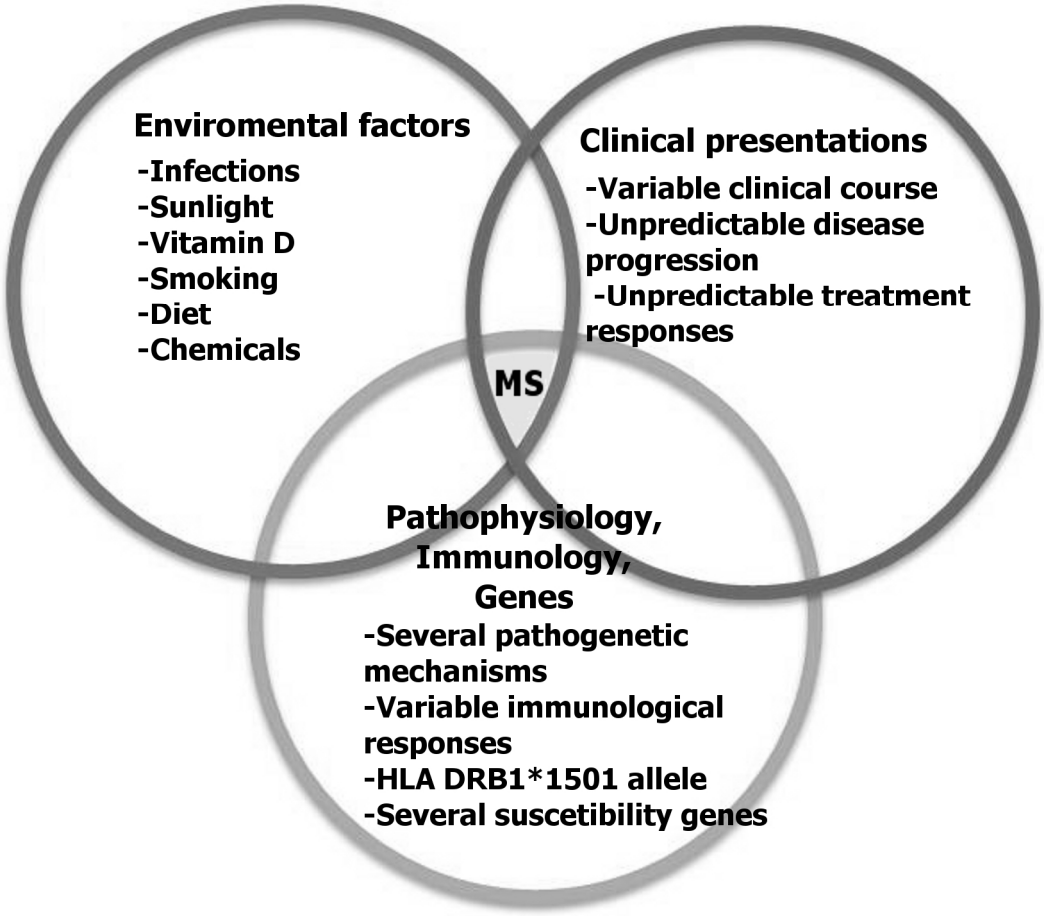


Figure 1. **Complex nature of multiple sclerosis.**

Environmental factors: Epidemiological and immigration studies have suggested that not only genetics factors, but also environmental factors increase the risk of developing MS (Kurtzke, 1993). Large-scale immigrant studies have shown that the risk of MS decreases, if children migrate from a high- to a low-risk region and the risk increase when children migrate from a low- to a high-risk region. Factors with the strongest evidence for involvement in MS are virus infections such as Epstein-Barr virus (EBV), the influenza A virus, smoking and vitamin D deficiency

(Milo and Kahana, 2010; Oikonen et al., 2011). Almost all individuals with MS (>99%) have been found to have been infected with EBV in comparison to age-matched controls (94%). MS is rare who have not been infected with EBV (Thacker et al., 2006; Ramagopalan et al., 2010). Sunlight exposure and vitamin D deficiency are the potential explanations for the associations between latitude and MS incidence. Sun exposure in childhood protects against MS (van der Mei et al., 2003; Islam et al., 2007; Kampman et al., 2007). Low serum levels of vitamin D are associated with a higher risk of developing MS (Munger et al., 2006; Lucas et al., 2011). Low vitamin D levels have been associated with an increased risk of relapses (Soilu-Hanninen et al., 2005; Smolders et al., 2008b; Simpson et al., 2010). Vitamin D has an immunoregulatory functions. It inhibits CD4+ T cell proliferation and the production of proinflammatory cytokines and stimulates production of anti-inflammatory cytokines (Smolders et al., 2008a). Smoking has been shown to increase the risk of MS and therefore promoting smoking cessation would be one of the most straightforward interventions available to reduce the incidence of MS (Hawkes, 2007).

Gender-related hormones: MS is more common in women than in men (Wallin et al., 2004; Orton et al., 2006; Alonso and Hernan, 2008; Hirst et al., 2009), but genome-wide studies have not found any MS-associated genes in the X chromosome. Therefore, the increased incidence of MS in women might be related with female-specific physiology and could thus be hormone related (Whitacre, 2001; Voskuhl, 2011). Sex hormones (estrogens, progestins, androgens) have been shown to modulate immune responses, as well as brain damage (Tomassini and Pozzilli, 2009). Especially estradiols and testosterone play an important modulatory role in MS brain damage.

Pregnancy provides another example of changes in hormonal patterns that influence clinical disease activity as well as subclinical disease activity. The Pregnancy in Multiple Sclerosis (PRISM) study was the first large prospective study that aimed at assessing the possible influence of pregnancy and delivery on the clinical course of MS (Vukusic et al., 2004). The study showed reduction in the relapse rate during pregnancy, especially in the third trimester, in comparison to the year before pregnancy. In the first three months postpartum the relapse rate increased compared to the pre-pregnancy state. Two years after delivery, the annualized relapse rate did not differ significantly from the pre-pregnancy rate. The effect of pregnancy

on MS disease activity has been explained by changes in the mother's endocrinal and immune system such as an increase in the levels of the sex hormones estrogen and progesterone together with a shift of in maternal immune responses from a prevailing Th1 to Th2 type responses (Nicot, 2009). A decrease in circulating NK cells and an increase in regulatory T cells (Treg) during pregnancy has been associated with reduced MS disease activity (Somerset et al., 2004; Saraste et al., 2007). Especially, an increase in circulating regulatory CD56(bright) NK cells and a decrease in circulating cytotoxic CD56(dim) NK cells in late pregnancy was considered to be associated with inhibition of MS activity during pregnancy (Airas et al., 2008).

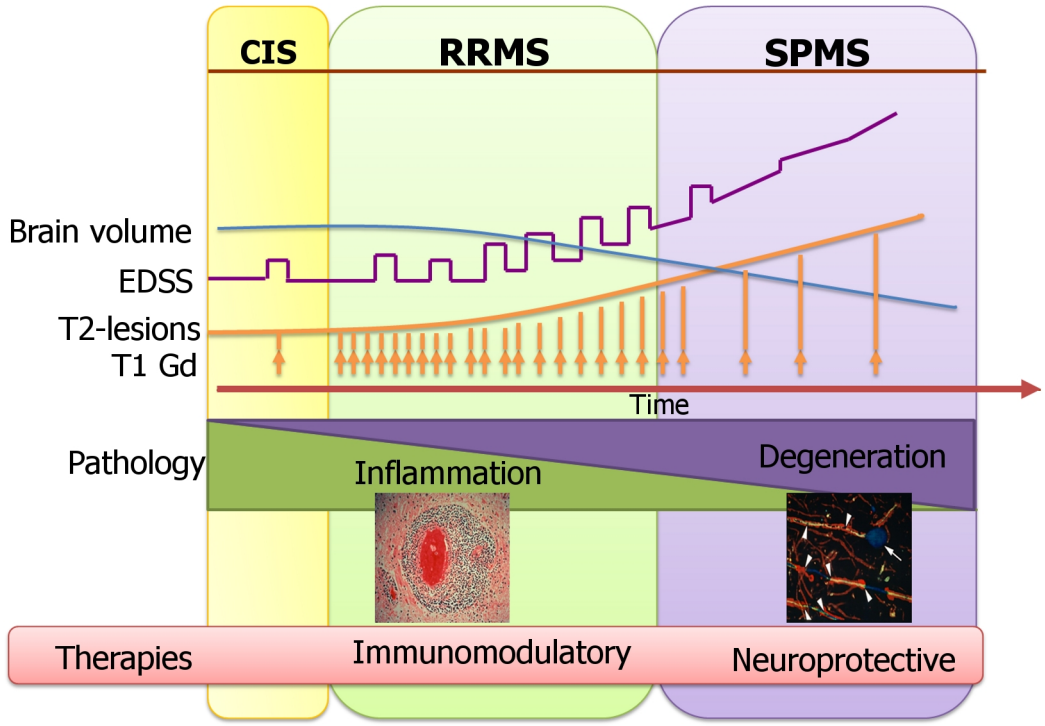


Figure 2. **Progression of MS from RRMS to SPMS.** Top: schematic representation of the disability progression expressed by expanded disability status scale (EDSS, violet line); frequency of inflammatory events when studied by magnetic resonance imaging: detection of Gadolinium (Gd)-contrast enhancing T1 lesions showing blood-brain barrier (BBB) opening (orange arrows); T2 lesion load reflecting brain tissue loss (orange line); brain volume indicative of brain atrophy (blue line). Pathology: in the early stage of the disease inflammation predominates whereas neurodegeneration is more pronounced at the later stages. On the left, perivascular inflammation with mononuclear cells, and on the right, axonal loss and demyelination. Therapies: in the early phase immunomodulatory therapies, which are targeted to decrease the inflammation are efficient, and in the later stage therapies, which protect the neurons would be beneficial. Redrawn from Sospedra and Martin 2005.

1.2 Clinical subtypes

MS has very heterogeneous clinical presentations and courses (Noseworthy et al., 2000). MS is classified on the basis of both the initial and the current clinical disease course (Figure 2 and 3). Most patients (85%) initially have RRMS, which is characterized by discrete clinical attacks or relapses followed by subsequent improvement. During these episodes of inflammation, clinical attacks typically develop over several days, become maximal after 1 to 2 weeks, and gradually resolve themselves over several weeks or months. RRMS is the most typical presentation in younger patients. After 10 to 20 years of RRMS, a majority of RRMS patients will enter a phase of progression with or without attacks, called SPMS. Figure 2 demonstrates disease development from RRMS to SPMS. A minority of patients (15%) have PPMS course from onset. These patients' disease progresses slowly without relapses. This subtype is considered to be a mostly noninflammatory subtype. (Lublin and Reingold, 1996; Compston and Coles, 2002)

The majority of patients with MS present in a relapsing-remitting fashion and their first attack presents as unilateral optic neuritis, a brainstem syndrome or partial myelitis. These presentations are known as clinically isolated syndrome (CIS) (Miller et al., 2005). Several placebo-controlled clinical trials on the efficacy of interferon (IFN)-β or glatiramer acetate (GA) have showed that majority of patients converted to MS during the two and three year follow-up (Jacobs et al., 2000; Comi et al., 2001; Kappos et al., 2006; Kinkel et al., 2006; Kappos et al., 2007; Comi et al., 2009)

1.3 Diagnosis

Diagnostic criteria for MS are mainly based on clinical evaluation, although this is facilitated by supportive laboratory and radiological investigations. During the last fifty years, various diagnostic criteria have been used (Schumacker et al., 1965; Poser et al., 1983; McDonald et al., 2001; Polman et al., 2005). In the beginning, the Schumacker criteria were based on clinical evidence of two relapses that were separated in space (lesions in the different locations in the CNS) and time (at least three months apart) (Schumacker et al., 1965). These criteria were updated in 1983

by the Poser criteria, which allow using also paraclinical evidence such as magnetic resonance imaging (MRI), oligoclonal band (OCB) analysis from cerebrospinal fluid (CSF) and visual evoked potential analysis (Poser et al., 1983). Eighteen year later the diagnostic criteria were updated by the McDonald criteria, which underline the importance of MRI in the diagnosis that allows earlier diagnosis of patients at their first episode (McDonald et al., 2001). The McDonald criteria were revised in 2005 and the changes mainly focus on the use of T2-weighted lesions and spinal cord imaging (Polman et al., 2005).

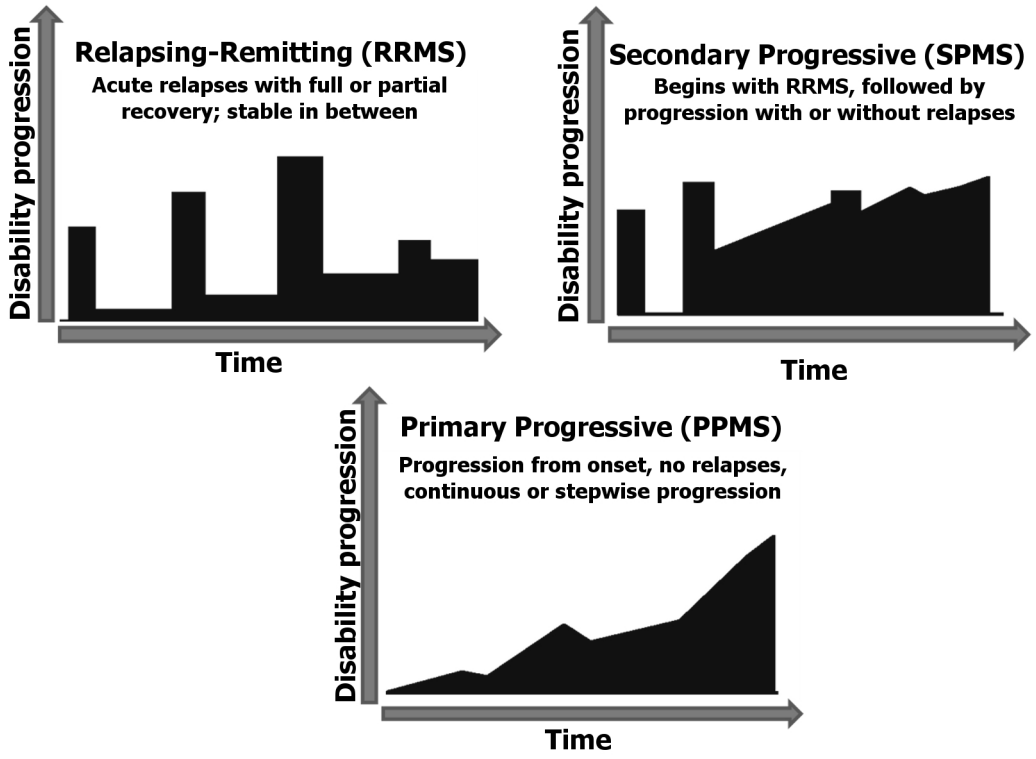


Figure 3. Clinical subtypes of MS. Redrawn from Lublin and Reingold 1996.

1.4 Disease-modifying therapies of multiple sclerosis

A number of disease-modifying therapies (DMT) with partial efficacy for patients with RRMS became available during the past 20 years**.** All of these drugs mainly target the inflammatory component of the diseases (Table 1). All the DMTs have shown to be effective in the RRMS type, but not in the PPMS. The most commonly used therapies that are approved for RRMS include the type-1 beta IFNs and GA (Table 1) (Barten et al., 2010). Several follow-up studies have evaluated the efficacy

of DMTs in MS, and it has been shown that treatments decrease the relapse rate and reduce the number of MRI lesions (The IFNB Multiple Sclerosis Study Group, 1993; Johnson et al., 1995; Jacobs et al., 1996; PRISMS Study Group, 1998). Also DMTs over a long period of time improves outcome by delaying the time to significant disease progression (Freedman, 2011).

Table 1. Current therapies for MS

Therapy	Mechanism of action	Developmental phase	Ref
First-line therapy			
Interferon-β	Inhibit T-cell activation and reduce blood-brain barrier (BBB) permeability to inflammatory cells.	Approved therapy for MS	1
Glatiramer acetate	Immune modulator and it is thought to shift the immune responses from T helper (Th) 1 to Th2 type.	Approved therapy for MS	2
Second-line therapies			
Natalizumab	Blocks very late antigen-4 (VLA-4) on the surface of lymphocytes and therefore reduce the transmigration of inflammatory lymphocytes via BBB into the CNS.	Approved therapy for MS	3
Mitoxantrone	Immunosuppressive cytotoxic agent that inhibits B-cell, T-cell, and macrophage proliferation and impairs antigen presentation and production of proinflammatory cytokines.	Approved therapy for MS	4
Fingolimod	Blocks sphingosine-1-phosphates receptors in T cells that results in an inhibition of T-cell migration from lymphoid tissue into the peripheral circulation and CNS.	Approved therapy for MS	4
Promising new drugs			
Cladribine	Purine nucleoside analog that produces selective lymphocyte depletion through the inhibition of cellular DNA synthesis and repair.	Phase III completed	5
Laquinimod	Immunomodulatory molecule that favors Th2/Th3 cytokine production and inhibits immune cell transmigration into the CNS.	Phase III ongoing	5

Teriflunomide	Inhibitor of dihydroorotate dehydrogenase, which is an integral membrane protein for pyrimidine synthesis. Inhibition of the enzyme prevents the clonal expansion of B and T cells and antibody production.	Phase III completed	4
Dimethyl fumarate	Anti-inflammatory and neuroprotective effects	Phase III	6
Alemtuzumab	Humanized monoclonal antibody directed against CD52 antigen on T cells and B lymphocytes, monocytes, macrophages, natural killer cells.	Phase III	6
Rituximab	First generation monoclonal antibody against the CD70 antigen on B cells that effectively depletes B cells via complement-dependent cell lysis and antibody dependent cellular toxicity.	Phase II/III	7
Ocrelizumab	Humanized monoclonal antibody against CD70.	Phase III	8
Ofatumumab	Fully human monoclonal antibody against CD70 antigen.	Phase III	8
Daclizumab	Monoclonal antibody directed against α subunit of interleukin-2 receptor (CD25) on activated T cells, which limits T-cell activation.	Phase III	6

1) Markowitz, 2007; 2) Farina et al., 2005; 3) Steinman, 2005; 4) Nicholas et al., 2011; 5) Fox, 2010; 6) Lim and Constantinescu, 2010; 7) Buttmann and Rieckmann, 2008; 8) Buttmann, 2010.

IFNs are the cytokines that are normally released from the lymphocytes in response to pathogens. There are three types of interferons: IFN-α, -β and -γ. IFN-β is approved for the treatments of MS. Its mechanism of action is not fully understood, but it has been shown to inhibit T-cell proliferation, reduce T-cell migration from the periphery to the CNS, and alter the T cell cytokine secretion toward anti-inflammatory responses (Markowitz, 2007). In clinical practice three different IFN-β preparations are available, in which dose and frequency of administration differ.

GA is a synthetic polypeptide (40-100 amino acids) composed of random sequences of four amino acids (tyrosine, glutamate, alanine, and lysine), which are common in myelin basic protein (MBP). GA clinical efficacy has been explained by a shift of proinflammatory responses towards anti-inflammatory responses (Farina et al., 2005; Johnson, 2010).

Currently, several potential therapeutic agents are being examined in ongoing phase II and III clinical trials (Table1). For example Alematzumab, Daclizumab and Rituximab are monoclonal antibodies targeting CD52+ T cell, CD25+ NK and T cells and CD20+ B cells, respectively (Buttmann and Rieckmann, 2008; Barten et al., 2010; Nicholas et al., 2011).

2. Pathogenesis and neuropathology

2.1 Neuropathology of multiple sclerosis

Pathologically MS is characterized by the presence of large, multifocal, demyelinated CNS lesions in multiple areas leading to scar tissue called sclerosis. This demyelination process is accompanied by autoimmune inflammatory reactions that are mediated mainly by T cells, B cells and macrophages. Primary targets are the myelin sheaths and oligodenrocytes, but axons, nerve cells and astrocytes are also affected (Frohman et al., 2006; Trapp and Nave, 2008).

The lesions observed in the CNS of MS patients are typically characterized by a demyelinated core separated by a very sharp border from normally myelinated surrounding tissue (Frohman et al., 2006). Many white matter lesions are detected in certain predilection sites, notably around the ventricles. Other predilection sites include the optic nerve and spinal cord. Moreover, lesions may also occur in the deep white matter far from ventricles as well as grey matter (Lassmann et al., 2007). The white matter lesions can be characterized broadly as chronic and active lesions. In chronic lesions there are less mononuclear cells, almost complete demyelination and severe astrogliosis. Active lesions are defined by ongoing destruction of myelin and are heavily infiltrated by macrophages and microglial cells (Frohman et al., 2006).

Clinical and MRI data suggest that inflammation and formation of new white matter lesions are the substrate for RRMS. In the progressive phase the new inflammatory demyelinating lesions are rare, but diffuse atrophy of the grey and

white matter and changes in the normal-appearing white matter (NAWM) are more predominant (Lassmann et al., 2007). Although MS is considered to be inflammatory demyelinating disease of the CNS, axonal injury and loss also occurs in the MS lesions and is associated with the development of permanent disability in MS patients (Trapp et al., 1998; Bjartmar et al., 2000). This suggests that progressive axonal loss may induce transition from RRMS to SPMS. Axon pathology and the frequency of transected axons in MS lesion correlate with the degree of inflammation (Frischer et al., 2009).

Normal appearing white matter may consider highly abnormal in MS patients, especially in patients in the progressive stage of the disease (Allen and McKeown, 1979; Kutzelnigg et al., 2005). Changes in the NAWM consist of a diffuse inflammatory process. Inflammatory infiltrates are present in the perivascular space and are also dispersed throughout the tissue. Inflammation is associated with profound microglia activation. This microglia activation is associated with diffuse axonal injury and loss (Kutzelnigg et al., 2005). The mechanisms that lead to diffuse injury of the NAWM are so far poorly understood. It is suggested that free radical mediated mitochondrial injury may be an important factor driving progressive axonal dysfunction and loss in MS (Lassmann et al., 2007).

Grey matter: MS lesion can also be located in the grey matter, especially in the cerebral cortex. Cortical lesions have been observed 80% of patients with PPMS. The histopathological characteristics of these cortical lesions differ substantially from white matter lesions (Calabrese et al., 2010). Three types of cortical lesions have been reported. Two of these lesions may appear in continuity with subcortical white matter plaques (type I) or as small intracortical perivascular lesions (type II). The most abundant form of cortical demyelination is subpial demyelination, which appears as large band-like lesions extending from the outer surface of the cortex into its deeper layer (type III) (Peterson et al., 2001).

Although cortical lesions are characterized by substantial loss of oligodendrocytes and axons (Peterson et al., 2001; Albert et al., 2007), they differ markedly from white matter lesions in terms of the degree and type of inflammation. Pure intracortical lesions typically have a very low degree of inflammation (Peterson et al., 2001; Bo et al., 2003; Geurts et al., 2005; Kutzelnigg et al., 2005). Perivascular infiltrates are rarely found in the MS cortex, and the density of infiltrating lymphocytes in pure cortical lesions is similar to the density of infiltrating lymphocytes in normal

appearing grey matter. Therefore, in cortical lesions demyelination processes may not be solely immune-mediated.

2.2 Immunopathogenesis of multiple sclerosis

2.2.1 Activation of immune cells

The cause of MS is still unknown and its pathogenetic pathways are not fully understood. Studies of experimental autoimmune encephalomyelitis (EAE), an animal model for MS, have indicated the role of myelin specific CD4+ Th1 and Th17 as the driving force in the autoimmune processes, but other cell types that contribute to the pathogenesis of MS, such as CD8+, B cells, or NK cells have also been investigated (Batoulis et al., 2010).

The initial step in immune cell activation is to activate dendritic cells (DC) via toll like receptors, which recognize very specific microbial products. After ligation, the DCs are activated and these cells start to produce type I IFNs. Once activated, CD4+ T cells interact with DC through the HLA class II molecule that recognizes the T-cell receptor (TCR) on the T cells (signal 1) in the secondary lymphoid organ (Figure 4 and 5). However, activation of T cells also requires additional signals. Interaction with HLA and TCR induces activation of CD40 ligand (CD154) on the surface of T cells, which binds to it receptor CD40 on the DC. This interaction induces upregulation of CD80 (B7-1) and CD86 (B7-2) on the surface of DC that interacts with CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA4) on the surface of the T cells (Signal 2). CD28 is associated with the activation of T cells, whereas CTLA4 (CD158) is more regulatory. After signal 2, DC start to produce important cytokines, which binds to receptors on the cell surface of T cells and drive them to secrete different cytokines. For example, secretion of IL-10, IL-12, IL-4 or combination of Interleukin (IL)-6/IL-1/IL-23 promotes differentiation to Th3, Th1, Th2, and Th17, respectively. (Kasper and Shoemaker, 2010)

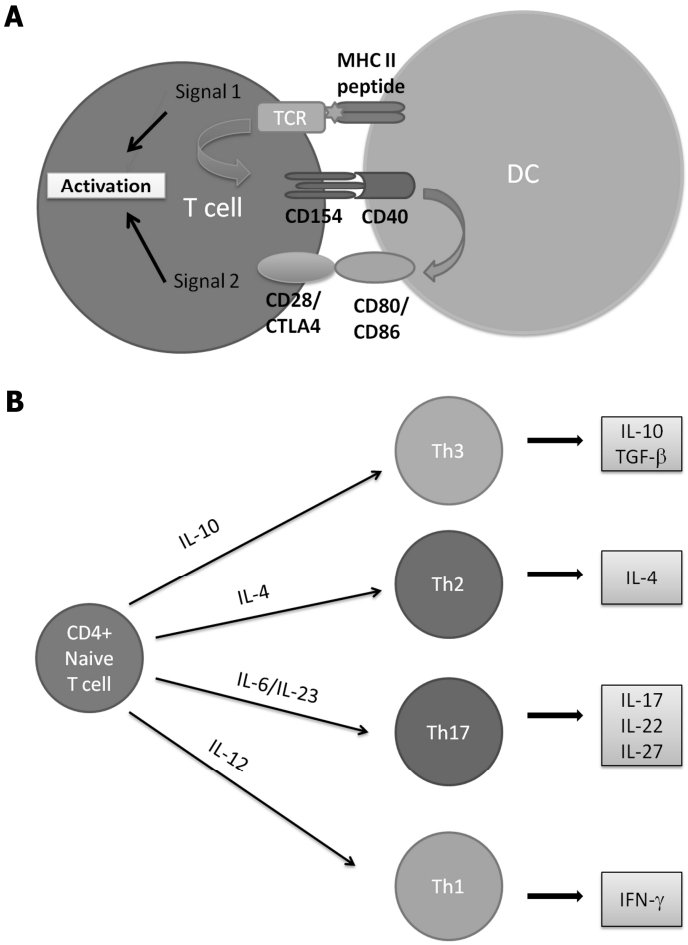


Figure 4. **Activation and differentiation of the naive CD4+ T cells. A.** On activation, the dendritic cells (DC) interact with T-cell receptor (TCR) on the surface of the T cells (signal 1). This induces activation of CD40 ligand (CD154) on the surface of the T cell, which binds its receptor CD40 on the DC. Then, upregulation of CD80 and CD86 on the surface of the DCs are induced. These molecules then interact with CD28 and cytotoxic T-lymphocyte antigen-4(CTLA4) on the surface of the T cells (signal 2), which finally activates T cell and leads to secretion of cytokines by the DC. **B.** Naive CD4+ T cells differentiation depends on the cytokine mileu. Secretion of Interleukin (IL)-10, IL-4, IL-12 or a combination of IL-6 and IL-23 promotes differentiation to T helper (Th) 3, Th2 and Th1 and Th17, respectively. **Abbreviations:** IFN interferon; MHC major histocompatibility complex; TGF transforming growth factor. Redrawn from Kasper and Shoemaker, 2010 and Fletcher et al., 2010.

2.2.2 Transmigration of immune cells to the central nervous system

Peripherally activated T cells enter distinct CNS microenvironments via the blood-brain barrier (BBB) within the CNS parenchyma and by blood-CSF barrier (BCB) within choroid plexus (figure 5). Tight junctions between endothelial cells of the

BBB and the epithelial cells of the BCB limit immune cells access to the CNS (Holman et al., 2011). However, activated and memory T cells can transmigrate into the CNS, because they express adhesion molecules, chemokine receptors and integrins that enable these cells to readily cross these barriers (Engelhardt et al., 1998; Engelhardt, 2008). The migration of leukocytes from blood into the CNS includes chronological leukocyte-endothelial interactions. Tethering and rolling of the leukocytes on the vascular endothelial surface initiate this process that is mediated by selectins and carbohydrate counter receptors. During the rolling, leukocytes also interact with the chemokines, which are produced on the endothelial surface. Chemokine stimulation induces upregulation of adhesion molecules of the integrin family on the leukocyte surface and leads to firm adhesion of leukocytes to the vascular endothelium by binding to cell adhesion molecules or the extracellular matrix component. This process ultimately leads to leukocyte transmigration to the CNS (Prendergast and Anderton, 2009).

2.2.3 Mechanisms of neural tissue damage

In the CNS, CD4+ T cells are reactivated and then mediate activation of macrophages/microglia, B cells and cytotoxic CD8+ T cells (Figure 5). Tissue damage in the inflammatory lesion of the brain and spinal cord may be induced by numerous mechanisms, such as cell-, cytokine, antibody- and radicals-mediated mechanisms (Lassmann, 2010).

Macrophage/microglia-mediated damage: Activated macrophages and microglia have an important role in the CNS lesion development. They produce a large array of toxic molecules, such as proteo- and lipolytic enzymes, cytotoxic cytokines, excitotoxins and reactive oxygen species or nitric oxide (NO) intermediates, which are potential inducers of axonal damage. For axonal injury, oxygen and NO radicals seem to be of particular importance (Jack et al., 2005; van Horssen et al., 2011).

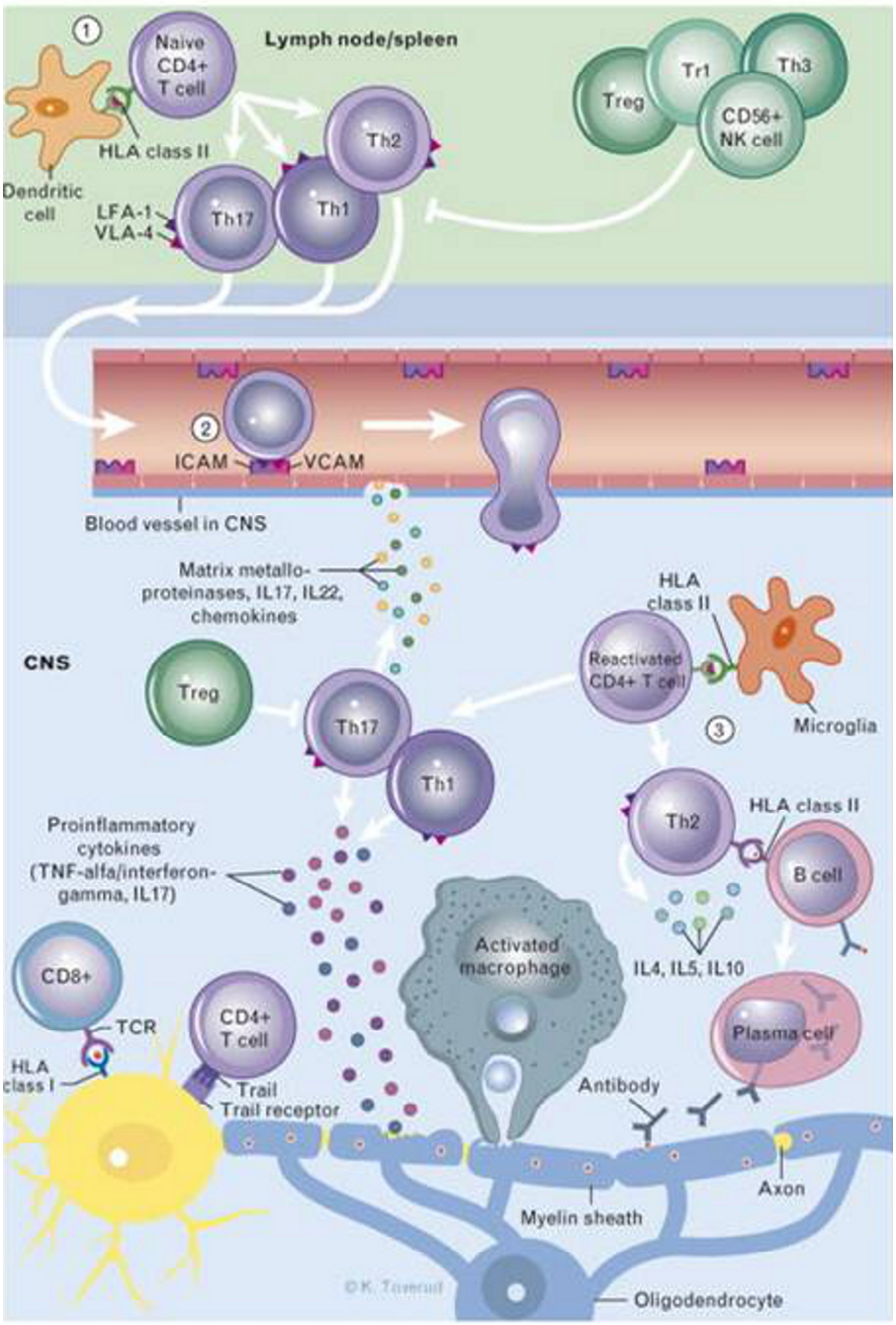


Figure 5. **Current understanding of immunological evens in MS.** 1) Autoreactive T cells are activated in secondary lymphoid tissue; 2) Activated T cells express adhesion molecules which enables them to transmigrate into the central nervous system (CNS); 3) Within the CNS, myelin specific T cells are reactivated and start to produce cytokines, like T helper (Th) 1 and Th17 cells produce tumor necrosis factor (TNF)-α and interferon (IFN)-γ, which may damage oligodendrocytes and neurons. Th2 cells secrete anti-inflammatory cytokines, which provide help to B cell-mediated tissue damage. **Abbreviations:** HLA human leukocyte antigen; LFA lymphocyte function associated antigen; VLA very late antigen; NK natural killer cells; Treg regulatory T cells; ICAM intracellular adhesion molecule: VCAM Vascular cell adhesion protein 1; IL interleukin. Picture adopted from the article by Holmoy and Hestvik, 2008, reproduced with permission of the publisher.

T cells are also central to MS lesion development. Both CD4+ and CD8+ T cells can express ligands for death receptors. Among them, TNF-related apoptosis inducing ligand (TRAIL), Fas ligand (FasL), tumor necrosis factor (TNF)-α have been described to mediate neuronal and oligodendrocytes damage (Aktas et al., 2006). CD8+ T cells are more prevalent in the MS lesions than the CD4+ T cells, and CD8+ cells can recognize antigen presented on HLA class I on neurons and oligodendrocytes and kill the target cells (Hauser et al., 1986; Friese and Fugger, 2009).

B cells and antibodies, B cell derived plasma cells and antibodies are found in the CNS of MS patients (Baranzini et al., 1999). The target antigens of the humoral immune response in MS are still largely unknown, but it is suggested that the primary targets of the antibodies are the proteins of the myelin sheath, such as MBP, myelin oligodendrocytes glycoprotein (MOG) and proteolipid protein (PLP). Possible mechanisms are the antibody-mediated complement activation and antibody-dependent cellular cytotoxicity via Fc-receptors (Franciotta et al., 2008; Weber et al., 2011)

Pathological analyses of actively demyelinating lesions have revealed four different structural and immunological features. Separate pattern of demyelination were homogenous in multiple lesions of the same patients, but different between the patients (Lucchinetti et al., 2000) (Figure 6). In these lesions, activated macrophages or microglia, cytotoxic cytokines, reactive oxygen and nitrogen species or specific demyelinating antibodies and activated complement component could be found. Although all actively demyelinating lesions were associated with an inflammatory infiltrates composed mainly of T lymphocytes and macrophages, they segregated into the four different patterns (Pattern I-IV) based on the distribution of myelin protein loss, the plaque geography and extension, pattern of oligodendrocyte destruction and the immunological evidence of immunoglobulins and activated complement deposit. Demyelination may be induced by macrophages and their toxic products (pattern I), by specific demyelinating antibodies and complement (pattern II), by degenerative changes in distal processes, in particular periaxonal oligodendrocytes followed by apoptosis (pattern III) or by primary degeneration of oligodendrocytes followed by myelin destruction (pattern IV).

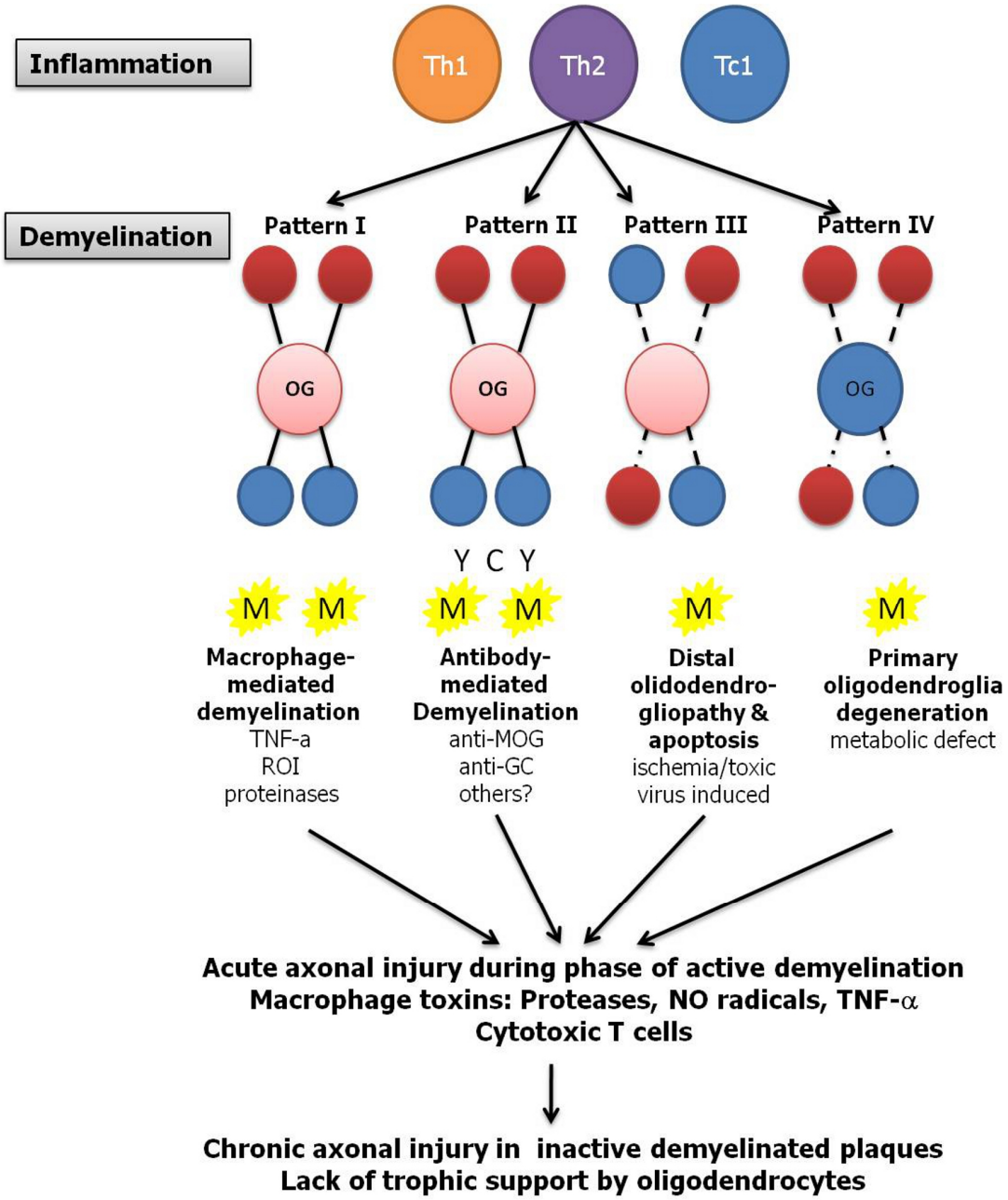


Figure 6. **Summary of pathogenetic mechanism of formation of actively demyelinating lesions.** Blue spheres indicate normal myelin sheath and red spheres demyelinated myelin sheath. Inflammation: T helper (Th) 1 cells maintain the inflammation in the central nervous system (CNS). These cells secrete proinflammatory cytokines that induce activation of macrophages (M), which are mainly responsible for the demyelination and axonal injury. Also Th2 and cytotoxic class I-restricted T cells (Tc1) might modify the outcome of the lesions. Demyelination: myelin sheath and oligodendrocytes (OG) are destroyed with multiple mechanisms. Four different mechanisms (pattern) in active demyelinating lesions have been found. Axonal injury: axonal injury follows acute destruction of myelin sheath. **Abbreviations:** C complement; GC galactocerebroside; M macrophages; MOG myelin oligodendrocytes glycoprotein; NO nitric oxide; ROI reactive oxygen species; TNF tumor necrosis factor; Y antibody. Redrawn from Lassmann et al., 2001.

2.2.4 Apoptosis

Once T cells have been activated and the invading antigen has been eliminated, T cells must be eliminated to maintain homeostasis. Some of T cells develop into the memory T cells and these cells are resistant to death by apoptosis. T cell survival is dependent on the signals, which cells receive through the TCR and costimulatory molecules, such as CD28, adhesion molecules and cytokines and through pro- and anti-apoptotic molecules (Krammer et al., 2007). Apoptosis can be triggered by two mechanisms: activation-induced cell death (AICD) and activated T cell autonomous death (ACAD). In AICD, apoptosis is induced when activated T cells are restimulated via TCR in the absence of appropriate co-stimulation. Then apoptosis can be triggered by an intrinsic or an extrinsic mechanism. However, activated T-cell death by ACAD does not require restimulation of TCR, and it is mediated by the intrinsic pathway. ACAD is also known as death by neglect, death by cytokine withdrawal or passive cell death (Brenner and Mak, 2009). T-cell apoptosis may be induced by several mechanisms: by extrinsic cell-death-receptor and caspase-dependent apoptosis, by intrinsic mitochondria- and caspase-dependent apoptosis, or by caspase-independent cell death. (Krammer et al., 2007)

In MS, a failure in the apoptosis cell death of autoreactive T cells is an important pathophysiological mechanism. Apoptosis is an important mechanism in immune system regulation in relation to the elimination of autoreactive T and B cells and macrophages from the circulation preventing their entry into the CNS (Pender, 2007). In EAE, T-cell apoptosis occurs in the CNS in acute EAE and plays an important role in the spontaneous recovery from disease. The main site of T-cell apoptosis is the brain parenchyma rather than perivascular space or meninges (Pender et al., 1991; Schmied et al., 1993; Tabi et al., 1994; Bauer et al., 1998).

The intrinsic apoptotic pathway is regulated by the balance between proapoptotic and antiapoptotic members of the B-cell lymphoma 2 (BCL-2) family, and it crucially depends on permeabilization of the outer mitochondrial membrane (Figure 7B). This pathway can be triggered by several stimuli, including TCR stimulation, UV-irradiation, DNA damage, endoplasmic reticulum stress, hormones and cytokine deprivation. Mitochondrial membrane permeabilization results in the release of mitochondrial content, including molecules such as cytochrome c. Release of cytochrome c induce formation of apoptosome that leads to activation of initiator

caspase-9, which then activates downstream effector caspases (Krammer et al., 2007).

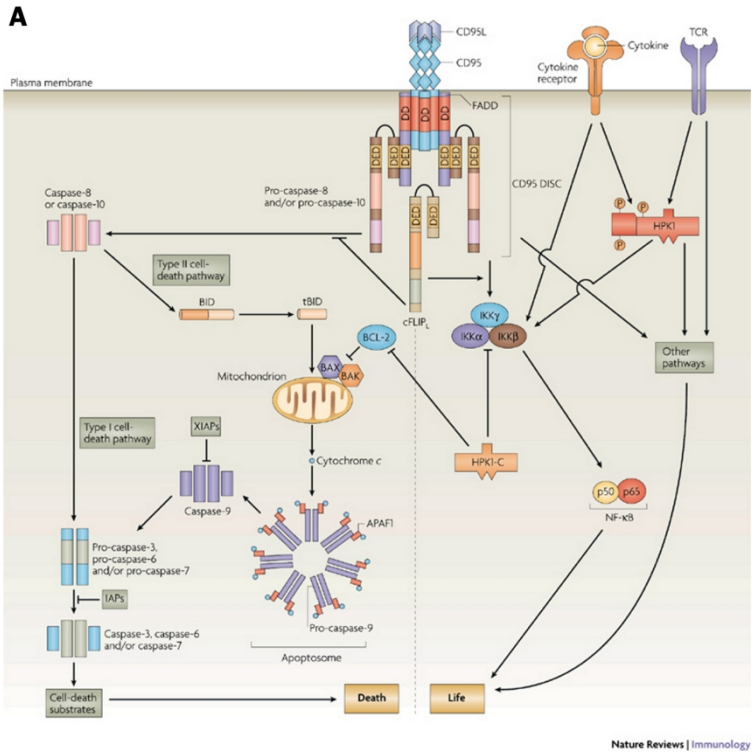
BCL-2 family members have both pro- and antiapoptotic functions, and these proteins are divided into three subgroup based on their structure (Table 2). All of these proteins contain between one to four distinct regions of homology, which are known as BH domains (Cepok et al., 2009). Antiapoptotic BCL-2 proteins contain four BH domains (BH1-4) and are generally integrated mostly within the outer mitochondria membrane. BCL-2, A1, BCL-XL and MCL-1 are the most important members of anti-apoptotic BCL-2 family and their function is to inhibit proapoptotic BCL-2 protein from permeabilization of the outer mitochondria membrane and release cytochrome c. (Sattler et al., 1997; Chipuk et al., 2010)

Table 2. BCL-2 family members

Class	Members	Structure
Anti-apoptotic BCL-2 family proteins	A1, BCL-2, BCL-XL, MCL-1	BH1-BH4
Pro-apoptotic effector proteins	BAX, BAK, BOK	BH1-BH3
Pro-apoptotic BH3-only proteins	BID, BIM, BAD, BIK, BMF, HRK, NOXA, PUMA	BH3

Abbreviations: BCL B-cell lymphoma; MCL-1 Induced myeloid leukemia cell differentiation protein; BAX Bcl-2–associated X protein; BAK Bcl-2 homologous antagonist/killer; BOK Bcl-2-related ovarian killer protein; BID BH3 interacting domain death agonist; BIM Bcl2-interacting mediator of cell death; BAD BCL2-associated agonist of cell death; BIK BCL2-interacting killer (apoptosis-inducing); BMF Bcl2 modifying factor; HRK harakiri, BCL2 interacting protein (contains only BH3 domain); NOXA Phorbol-12-myristate-13-acetate-induced protein-1; PUMA p53 up-regulated modulator of apoptosis.

BCL-2 proapoptotic members are divided into the effector proteins and BH3-only proteins. The effector proteins BAK and BAX homo-oligomerize into proteolipid pores within the outer mitochondria membrane and induce outer mitochondria membrane permeabilization (Mikhailov et al., 2003). BH3-only proteins function is to interact with the antiapoptotic BCL-2 family members and trigger apoptosis when overexpressed. BIM and PUMA can bind antiapoptotic BCL-2 and BCL-XL, which induces inhibition of BAK and BAX and results in cytochrome c release and induction of cell death (Bouillet and Strasser, 2002). Therefore, the ratios of antiapoptotic to proapoptotic BCL-2 family members determine whether cells die or live (Chipuk et al., 2010).


A
CD95L
CD95
Plasma membrane
FADD
DD
DED
Cytokine
Cytokine receptor
TCR
CD95 DISC
Caspase-8 or caspase-10
Pro-caspase-8 and/or pro-caspase-10
HPK1
P
Type II cell-death pathway
BID
tBID
cFLIP$_L$
IKKγ
IKKα
IKKβ
BCL-2
BAX
BAK
Mitochondrion
Other pathways
Type I cell-death pathway
XIAPs
Cytochrome c
HPK1-C
Caspase-9
p50
p65
NF-κB
APAF1
Pro-caspase-3, pro-caspase-6 and/or pro-caspase-7
IAPs
Pro-caspase-9
Caspase-3, caspase-6 and/or caspase-7
Apoptosome
Cell-death substrates
Death
Life
Nature Reviews | Immunology

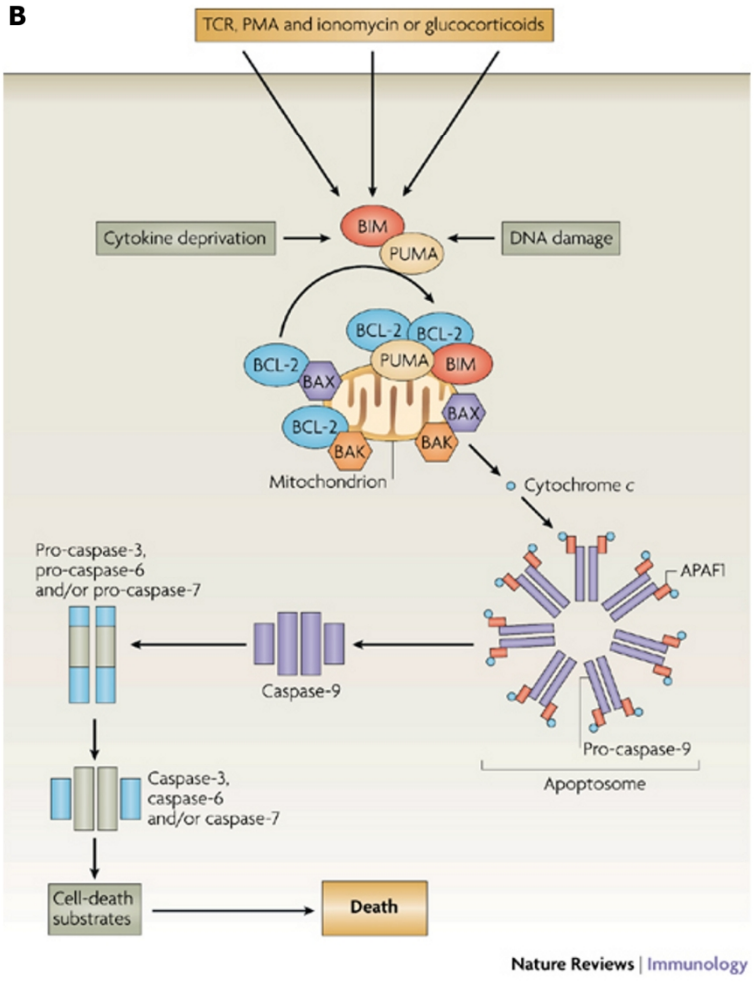

B
TCR, PMA and ionomycin or glucocorticoids
Cytokine deprivation
BIM
PUMA
DNA damage
BCL-2
BAX
BAK
Mitochondrion
Cytochrome c
APAF1
Pro-caspase-3, pro-caspase-6 and/or pro-caspase-7
Caspase-9
Pro-caspase-9
Apoptosome
Caspase-3, caspase-6 and/or caspase-7
Cell-death substrates
Death
Nature Reviews | Immunology

Figure 7. **Apoptosis can be triggered by two main pathways. A)** The extrinsic pathway is mediated death factors, like Fas ligand (FasL), tumor necrosis factor (TNF) and TNF-related apoptosis-inducing ligand (TRAIL). These death factors induce the apoptosis signal cascade via death inducing complex (DISC). **B)** Mitochondrial damage, due to anticancer drugs, γ-radiation, antioxidants, growth factor or serum deprivation, leads to cytocrome c release from mitochondria into to the cytosol. Cytocrome c can activate caspase 9 together with adaptor apoptotic protease activating factor 1 (Apaf-1) and thus activates effector caspases. This pathway is also called the intrinsic pathway. Picture adopted from the article by Krammer et al., 2007, reproduced with permission of the publisher.

In the extrinsic apoptotic pathway, proapoptotic signals are triggered by signals emanating from the cell-surface death receptors (DR) that are activated by ligands, such as TNF, FasL and TRAIL (Figure 7A). DRs are defined by the presence of a death domain (DD), which is essential for the apoptosis induction (MacEwan, 2002).

The transduction of the apoptotic signals from the cell death receptors starts with the formation of a large protein complex at the cell membrane, known as the death-inducing signaling complex (DISC), in which activation of caspase-8 and caspase-10 takes place. Activated caspases-8 and caspase-10 directly lead to the activation of downstream effector caspases caspase-3, caspase-6 and caspase-7 that lead to cell death. Sometimes formation of DR-DISC is reduced leading to lower levels of active caspase-8. In these cases apoptosis is induced by cleavage of BCL-2 family protein BID, which interacts with BAX or BAK. This interaction induces the release of cytochrome c from mitochondria that results in the formation of a multi-protein complex apoptosome, which can lead to the activation of caspase 9, and then downstream downstream effector caspases (Krammer et al., 2007).

2.3 Cytokines and chemokines

2.3.1 Cytokines

Cytokines are soluble proteins or glycoproteins that mediate the pathogenesis of MS. Constitutive production of cytokines is usually low or even absent and they are generally produced in small quantities in response to local stimulus, such as the presence of antigen or endotoxin, or the transduction of signal provided by other cytokines (Ozenci et al., 2002).

It is generally known that IFN-γ, TNF-α and IL-6 play an important role in MS pathogenesis (Table 3). Elevated levels of IFN-γ have been detected in the CNS at the peak of EAE disease and treatment of patients with IFN-γ was deleterious to MS patients (Panitch et al., 1987). Also elevated levels of TNF-α have been detected within MS lesions (Hofman et al., 1989). TNF-α is also present in higher amount in the serum and CSF of MS patients compared to healthy controls and it also correlates with the severity of the lesions and disease progression (Maimone et al., 1991; Sharief and Hentges, 1991). IL-6 has been detected in chronic MS lesions and IL-6 deficient mice were resistant to EAE (Okuda et al., 1999; Okuda et al., 2000). Previously, IL-6 was believed to mediate the generation of Th1 cells and therefore modulate the Th1/Th2 balance. Recently, IL-6 together with IL-23 have been shown to be essential factors required for the expansion of a pathogenic CD4+ T cell population to IL-17 producing Th17 cells (Lovett-Racke et al., 2011). Until now, the IL-23/Th17 pathway has been thought play an important role in MS. IL-17 is elevated in the active MS brain lesions (Lock et al., 2002; Tzartos et al., 2008). Th17 cells have shown to migrate efficiently through a model of the BBB, accumulate in MS lesions and secrete granzyme B and other cytolytic enzymes that kill neurons (Kebir et al., 2007). A higher number of IL-17 positive mononuclear cells in blood and CSF were detected compared to healthy controls. Increased levels of IL-17 have been found in mononuclear cells in MS patients during exacerbation and it decreased in response to high dose of IFN-β and GA (Durelli et al., 2009).

Similarly to TNF-α, other cytokines of the TNF ligand superfamily participate in immune cell activation and differentiation, as well as maintaining the homeostasis of the immune system via apoptosis. The best characterized of these ligands are TNF-α, FasL and TRAIL. They are membranous proteins that are also cleaved from the cell membrane by the metalloproteinases (Tanaka et al., 1998; Bossi and Griffiths, 1999; Croft, 2009).

Fas/FasL interaction is essential for T-cell homeostasis by deletion of autoreactive T cells. Membranous FasL primarily mediates apoptosis and soluble FasL can have a both pro- and antiapoptotic functions depending on the nature of the other appropriate mediators in the microenvironment. The antiapoptotic function is mediated by sFasL that competes with the membranous form of Fas binding. Sometimes sFasL can interact with extracellular matrix proteins that may potentiate

its proapoptotic activity (Aoki et al., 2001). In contrast to FasL, the soluble form of the Fas receptor is generated by alternative splicing of mRNA instead of proteolytic cleavage of membranous protein (Cascino et al., 1995). sFas have a negative regulatory effect on the membrane-bound Fas, because both forms can bind to FasL (Cheng et al., 1994).

In MS, several studies have detected upregulated levels of Fas and FasL in MS lesions indicating these molecules to be potential effectors of this disease (D'Souza et al., 1996; Dowling et al., 1996; Bonetti and Raine, 1997). Elevated Fas expression on oligodendrocytes in chronic active and chronic silent MS lesions compared to oligodendrocytes in control tissue from subjects with or without other neurological diseases has been found (D'Souza et al., 1996; Bonetti and Raine, 1997). FasL expression is localized to microglia cells and T cells. *In vitro*, FasL was shown to induce cell death of oligodendrocytes (D'Souza et al., 1996). Defective T cell Fas function in patients with MS has also been reported (Comi et al., 2000). This is also supported by studies that have reported upregulated level of sFas in sera and CSF of patients with RRMS patients, especially during clinical disease activity (Inoue et al., 1997; Zipp et al., 1998b; Boylan et al., 2001). Moreover, intrathecal production of sFas has been reported in MS (Ciusani et al., 1998). Furthermore, it was confirmed that sFas is biologically active via inhibition of FasL-mediated apoptosis in MS (Zipp et al., 1998a).

TRAIL (Apo2L, TNFRSF10) is abundantly expressed in immune cells, especially in the NK cells, NKT cells and macrophages. Also the soluble trimeric form is biologically active (Bodmer et al., 2000; Trabzuni et al., 2000). TRAIL is considered to play a crucial role in maintaining T-cell homeostasis, as well as killing tumor and virally transformed cells by NK and NKT cells. TRAIL is present at high levels in chronic and acute MS lesions, where the most relevant sources include activated microglia and invading immune cells (Cannella et al., 2007).

In EAE models, TRAIL has been shown to inhibit disease development (Hilliard et al., 2001; Cretney et al., 2005). *In vitro* studies have shown that recombinant TRAIL inhibits T-cell activation and proliferation, but does not induce apoptosis in the T cells (Song et al., 2000; Lunemann et al., 2002). In the CNS, TRAIL has been shown to mediate apoptosis of human neurons and oligodendrocytes (Nitsch et al., 2000; Matysiak et al., 2002). In the EAE model, intracerebral injection of soluble

TRAIL was shown to increase neuronal apoptosis and increase clinical deficits (Aktas et al., 2005).

Table 3. Cytokines described in MS

Ligand	Receptor	Produced by	Function in MS	Ref.
Predominantly proinflammatory				
IFN-γ	IFNGR1-IFNGR2	NK, γδT CD8+ Th1 CD4+	-Activates mononuclear cells, induces MHC I and MHC II expression, influences antibody production and apoptosis of T cells -Detected in the MS lesions and increased levels of IFN-γ are associated with disease exacerbation	1,2
TNF-α	TNFR1, TNFR2	MΦ, microglia astrocytes T cells	-Activates mononuclear cells, induces expression of adhesion molecules, chemokines and cytokines and triggers apoptosis of oligodendrocytes -Expressed in the active MS lesions and increased levels are associated with active disease	1,3,4
IL-17	IL17R, IL17Rβ	Th17 CD8+ NK NKT	-Induces secretion of proinflammatory cytokines and chemokines, activate microglia cells and mediate BBB disruption -Elevated in active MS brain lesions and increased levels associated with active disease	5, 6-8
IL-6	IL6Rα-gp130	T cells MΦ	-Induces differentiation of Th17 cells and B cell differentiation and immunolglobulin production -Detected in MS lesions, but no association with disease activity or progression	1, 3-9
IL-23	IL23R-IL12Rβ1	MΦ DC	-Efficient role in differentiation and survival of Th17 cells -Increased levels in RRMS patients	5, 10
IL-12	IL12Rβ1-IL12Rβ2	Monocytes DC	-Mediates differentiation of Th1 cells -Elevated in the acute MS lesions, increased in the RRMS and SPMS patients and correlate with development of active MS lesion	10-13
FasL	FAS	Microglia T cells NK	-Mediates apoptosis of immune cells and oligodendrocytes -Detected in MS lesions and increased levels of sFas were detected in some studies	14-17
TRAIL	TRAILR1, TRAILR2, DcR1, DcR2, OPG	NK NKT MΦ	-Inhibits T-cell activation and mediate apoptosis of neurons and oligodendrocytes -Expressed in the MS lesions and suggested as potential response marker for IFN-β therapy	18, 19

Predominantly regulatory				
IL-4	IL4Rα-IL4Rγ	Th2	-Mediates differentiation of Th2 cells and B cells -Detected MS lesions and increased levels detected during the relapse	1, 20, 21
IL-10	IL-10Rα-IL10Rβ	Monocytes MΦ B cells Th2	-Inhibits production of proinflammatory cytokines and T-cell proliferation -Decreased levels of IL-10 detected before exacerbation	1, 22
TGF-β	TGFBR1-TGFBR2	Treg Monocytes Astocytes Microglia	-Inhibits proliferation of T cells and macrophages, maturation of cytotoxic lymphocytes and NK cells -Decreased levels of TGF-β in blood detected in RRMS patients	1, 22

Abbreviations: IFN interferon; TNF tumor necrosis factor; IL interleukin; FasL Fas ligand; TRAIL TNF-related apoptosis inducing ligand; TGF transforming growth factor; R receptor; Dc decoy; OPG osteoprotegrin; MΦ macrophages; NK natural killer cells; NKT natural killer T cells; Th T helper cells; Treg regulatory T cells.
1) Imitola et al., 2005; 2) Traugott and Lebon, 1988; Hohnoki et al., 1998); 3) Cannella and Raine, 1995; 4) Spuler et al., 1996; 5) Jadidi-Niaragh and Mirshafiey, 2011;
6) Durelli et al., 2009; 7) Tzartos et al., 2008; 8) Lock et al., 2002; 9) Harris and Sadiq, 2009a; 10) Alexander et al., 2010; 11) Fletcher et al., 2010; 12) van Boxel-Dezaire et al., 1999; 13) van Boxel-Dezaire et al., 2001; 14) D'Souza et al., 1996;
15) Aktas et al., 2006; 16) Dowling et al., 1996; 17) Bonetti and Raine, 1997;18) Hoffmann et al., 2009; 19) Wandinger et al., 2003; 20) Bartosik-Psujek and Stelmasiak, 2005a; 21) Cannella and Raine, 1995; 22) Rieckmann et al., 1994.

2.3.2 Chemokines

Chemokines and their receptors play an important role of the recruitment of immune cells from the periphery into the CNS (Savarin-Vuaillat and Ransohoff, 2007). The chemokine family comprises approximately 50 ligands and 20 receptors in humans. Chemokines are small soluble (8-14 kDa) proteins that are classified into four main families based on the location of intramolecular disulphide bonds (CC, CXC, CX3C, CX) in the N-terminus. Chemokines bind to their corresponding seven-transmembranedomain G-protein coupled receptor on the target cells. Most of the chemokine receptors and ligand do not have specific pairs, but for instance ten different CC family ligands can bind to chemokine receptor CCR1. Conversely, some ligands can productively signal many receptors, such as CCL3 can signal with CCR5 and CCR1.(Moser et al., 2004)

In the MS pathogenesis several chemokines and chemokines receptors have been shown to be important (Table 4). For example, CCL2, CCL4, CCL5, CXCL10,

CXCL12, and CXCL13 and their receptors including CCR1, CCR2, CCR5, CXCR3, and CXCR4 have been detected in the active MS lesions, as well as in CSF and blood where they were considered to reflect disease activity (Szczucinski and Losy, 2007). Recently, expression of CXCR3+ on circulating CD8+ cells was associated with MRI measurements of inflammatory activity and tissue destruction (Fox et al., 2008). Interestingly, it has been reported that IFN-β can modulate the levels of several chemokines (CCL1, CCL2, CCL7, CXCL10 and CXCL11) in blood and CSF, thus limiting the entry of immune cells into the CNS (Cepok et al., 2009; Sellebjerg et al., 2009b).

Table 4. Chemokines described in MS

Chemokine	Chemokine receptors	Target cells	Role in MS	Ref.
CCL2 (MCP-1)	CCR2	Monocytes T cells NK	- Detected in the MS lesions by astrocytes and macrophages - Decreased expression of CCL2 in the CSF and blood of MS patients, especially during active disease	1-6
CCL3 (MIP-1α)	CCR1 CCR5	Monocytes	- Detected in actively demyelinating lesions by macrophages and microglia - Increased expression of CCL3 in the CSF during active disease	2,7
CCL4 (MIP-1β)	CCR5	Th1 Monocytes	- Detected in actively demyelinating lesions by macrophages and microglia - Not known in MS	2
CCL5 (RANTES)	CCR1 CCR5	Monocytes Th1	- Detected in actively demyelinating lesions by macrophages and microglia - Increased expression of CCL5 in the CSF and blood of MS patients , especially during active disease	2,8-10
CCL7 (MCP-3)	CCR1 CCR2	Monocytes	- Detected in the acute and chronic MS lesions by astrocytes and inflammatory cells - Studies of CCL5 in blood or CSF not available	1
CCL8 (MCP-2)	CCR3	Monocytes T cells	- Detected in the acute and chronic MS lesions by astrocytes and inflammatory cells - Studies of CCL8 in blood or CSF not available	1

CXCL8 (IL-8)	CXCR1 CXCR2	Monocytes	- Detected in active MS lesions by astrocytes - Increased secretion of CXCL8 in blood of MS patients	11-13
CXCL9 (MIG)	CXCR3	Th1	- Detected in actively demyelinating lesions by astrocytes and macrophages - Increased secretion of CXCL9 in CSF from active MS patients	10,14
CXCL10 (IP-10)	CXCR3	Th1	- Detected in actively demyelinating lesions by astrocytes (Simpson et al., 2000) - Decreased expression of CXCL10 in the CSF and blood of MS patients, especially during active disease	3-6, 15
CXCL12 (SDF-1)	CXCR4 CXCR7	Lympho-cytes MC	- Detected in active and chronic inactive lesions by astrocytes - CXCL12 were expressed constitutively in blood and CSF in MS	16,17
CXCL13 (BCA-1)	CXCR1	B cells	- Detectable in the active MS lesions by perivascular infiltrates - Increased secretion of CXCL13 in the CSF and blood of active MS patients	16-18

Abbreviations: CCL C-C motif chemokine ligand; CXCL C-X-C motif chemokine ligand; CCR C-C motif chemokine receptor; CXCR C-X-C motif chemokine receptor; NK natural killer cells; Th T helper cells.
1) McManus et al., 1998; 2) Simpson et al., 1998; 3) Franciotta et al., 2001; 4)Scarpini et al., 2002; 5) Sorensen et al., 2001; 6) Mahad et al., 2002; 7)Miyagishi et al., 1995; 8) Bartosik-Psujek and Stelmasiak, 2005a; 9) Sindern et al., 2001; 10) Sorensen et al., 1999; 11) Omari et al., 2005; 12) Lund et al., 2004; 13) Bartosik-Psujek and Stelmasiak, 2005b; 14) Simpson et al., 2000 ; 15) Balashov et al., 1999; 16) Krumbholz et al., 2006; 17) Sellebjerg et al., 2009a; 18) Festa et al., 2009.

3. Biomarkers in multiple sclerosis

Biomarkers are measurable indicators of normal biological and pathogenic processes, or pharmacological responses to a therapeutic intervention. A good biomarker should be precise and reliable, distinguishable between normal and MS disease. Measurements of biomarkers are traditionally limited to detection of specific proteins

in the body fluids (blood, CSF, urine) that become altered as a consequence of a biologic or pathologic process (Biomarkers Definitions Working Group., 2001; Bielekova and Martin, 2004).

Most MS lesions are typically located in the periventicular white matter of the brain as well as superficial areas of the spinal cord, which share a close anatomical relationship with CSF space (Tumani et al., 2009). Because brain biopsies are mostly unavailable, CSF is important sample in understanding the pathology of MS. CSF can be used for measurements of various soluble markers and cell populations by flow cytometry analysis, PCR studies and cell functional studies. However, CSF collection is an invasive procedure, and therefore it is collected only rarely, usually during the diagnostic procedure. (Bielekova and Martin, 2004)

Biomarkers that are measurable from the peripheral blood are of great clinical value because of the non-invasive collection method. The main disadvantage of blood is that the disease pathology is restricted to the CNS that is separated from the periphery by the BBB. Therefore, biological events that are associated with CNS lesions may not be measurable in the peripheral blood. Also, the diurnal variation of many soluble markers and levels of measured markers are often affected by systemic infections, by biological degradation in the liver or by excretion in the kidney. However, peripheral blood biomarkers can provide important information regarding immune triggers of MS, as well as the therapeutic efficacy of drugs administered systemically. (Bielekova and Martin, 2004; Harris and Sadiq, 2009b; Tumani et al., 2009) It is also noteworthy that the majority of protein content of the lumbar CSF is blood-derived and the remainder consists of less brain-derived or intrathecally produced proteins. Under physiological conditions blood-derived proteins enter into the CSF compartment via passive diffusion across the blood-CSF barrier (Tumani et al., 2009).

Pathological studies have revealed several mechanisms that are important in MS pathogenesis and therefore classification of process-specific biomarkers is based on these studies (Trapp et al., 1998; Lucchinetti et al., 2000). Thus, biomarkers can be categorized into the following classes: inflammation, BBB function, degeneration and regeneration (Table 5) (Bielekova and Martin, 2004).

3.1 Biomarkers of inflammation

Cytokines and cytokine receptors. Many cytokines and their receptors have been detected in the MS lesions and they are thought to play a role in the MS pathogenesis via immune system activation as well as via damaging neuronal cells. Proinflammatory cytokines have been studied extensively. CSF levels of proinflammatory cytokines are often elevated in MS patients (Rieckmann et al., 1994; Spuler et al., 1996; Fassbender et al., 1998; van Boxel-Dezaire et al., 2001; Bartosik-Psujek and Stelmasiak, 2005a; Malmestrom et al., 2006; Tzartos et al., 2008).Some studies have reported an association between increased cytokine levels in the CSF with the clinical disease activity and Gd-enhancing lesions in the MRI scan, but others could not confirm these findings (Spuler et al., 1996; Giovannoni et al., 2001).

Chemokines and chemokine receptors. Chemokines and their receptors play an important role in the recruitment of immune cells from the periphery to CNS and they have been detected in MS lesions (Szczucinski and Losy, 2007). CXCL13 has been shown to be upregulated in active MS patients and elevated levels might predict CIS conversion to CDMS (Festa et al., 2009; Sellebjerg et al., 2009a; Brettschneider et al., 2010; Khademi et al., 2011; Ragheb et al., 2011)

Antibodies. Intrathecal immunoglobulin synthesis and the presence of OCB in MS patients has been most the important and earliest evidence suggesting a role for B cells and antibodies in the pathology of MS (Awad et al., 2010). The presence of anti-myelin antibodies has been suggested to be a valuable marker for prognosis and progression in MS. The presence of serum IgM anti-MOG and anti-MBP antibodies has been shown to predict the risk of conversion from CIS to clinically definite MS in one study (Berger et al., 2003). Neuron-specific proteins are thought be important markers for MS. Anti-Neurofilament (NF) antibodies have been suggested to be markers of tissue damage in MS. Anti-NF antibodies are detected both in serum and CSF of MS patients and they are shown to correlate with brain parenchymal fraction, T2 and T2 lesion load, and MRI marker of tissue damage (Silber and Sharief, 1999; Eikelenboom et al., 2003). In another study serum anti-NF antibodies were significantly elevated in PPMS (Ehling et al., 2004).

Apoptosis-related molecules. Several studies have shown downregulation of pro-apoptotic molecules in active MS indicating abnormalities in the apoptotic cell death

of lymphocytes in MS (Sharief, 2000b; Semra et al., 2001; Sharief et al., 2002). Increased mRNA expression of Fas and FasL have been consistently reported in the peripheral blood mononuclear cells (PBMC) in RRMS, but the data on the sFas in sera is contradictory(Inoue et al., 1997; Zipp et al., 1998b; Huang et al., 2000; Bilinska et al., 2003; Gomes et al., 2003; Mahovic et al., 2004). Upregulated expression of TRAIL mRNA has been reported in PBMC of IFN-β treated RRMS patients who respond to treatment. Based on these observations it has been suggested that TRAIL could be used as a biomarker reflective of response to IFN-β treatment in MS. (Wandinger et al., 2003).

3.2 Biomarkers of blood-brain barrier function

Adhesion molecules. Transmigration of immune cells into the CNS is associated with BBB disruption and consecutive transendothelial migration of leukocytes that is mediated by adhesion molecules (Holman et al., 2011). Adhesion molecules are normally expressed at very low levels on vascular endothelial cells of normal brains, but the expression is upregulated after cytokine stimulation. Adhesion molecules also exist in soluble forms, released from the endothelial cells, immune cells and platelets. Soluble adhesion molecules sPECAM-1, sP-selectin and sE-selectin have been shown to be upregulated in RRMS patients when compared to PPMS and the levels of these molecules are also upregulated during relapse suggesting that these molecules might be used as a biomarker for disease activity (Kuenz et al., 2005).

Matrix metalloproteinases. MMPs are an important contributor to the inflammatory damage to BBB and myelin. Elevated level of MMP-9 correlated with Gd-enhancing lesions on MRI (Lee et al., 1999; Waubant et al., 2003).

3.3 Biomarkers of degeneration

Axonal and neuronal damage. Neurodegenerative biomarkers typically consist of neuron-specific proteins, which are released following axonal degeneration. Several studies have shown elevated CSF levels of NF in MS (Lycke et al., 1998; Malmestrom et al., 2003; Rejdak et al., 2008). Tau, protein of the axons, was found

to be upregulated in both RRMS and PPMS patients (Kapaki et al., 2000; Bartosik-Psujek and Archelos, 2004; Martinez-Yelamos et al., 2004; Bartosik-Psujek and Stelmasiak, 2006; Guimaraes et al., 2006; Terzi et al., 2007). Furthermore, CSF levels of Tau tended to be highest in the early stage of the disease (Martinez-Yelamos et al., 2004; Brettschneider et al., 2005).

Oxidative stress and exicitoxicity. Oxidative stress may responsible for CNS degenerative events. NO is a free radical with multiple biological effects, such as oligodendrocyte injury, axonal degeneration, and impairment of nerve conduction (Lassmann, 2010). Elevated levels of NO have been detected in acute demyelinating lesions. Elevated levels of NO metabolites nitrite and nitrate are associated with disease activity (Yamashita et al., 1997; Brundin et al., 1999; Svenningsson et al., 1999; Acar et al., 2003; Rejdak et al., 2004). NO synthase (NOS) was also increased in MS patients (Calabrese et al., 2002).

3.4 Biomarkers of regeneration

The expression of several neurotrophins has been observed in the MS lesions which can stimulate regeneration and promote repair. Neurotrophic factors are released mainly by T cells and other cells of the immune system.(Hohlfeld, 2008) Regeneration biomarkers have not been studied extensively. In MS patients, reduced levels of brain-derived neurotrophic factor (BDNF) have been reported in blood and CSF (Azoulay et al., 2005; Frota et al., 2009). The levels of BDNF, neural cell adhesion molecule (NCAM) and ciliary neurotrophic factor (CNTF) in the CSF of MS patients are associated with disease activity (Weinstock-Guttman et al., 2007; Frota et al., 2009).

Table 5. Summary of biomarkers in the multiple sclerosis. Modified from the Bielekova and Martin, 2004.

Biomarker	Predominant origin	Description	Usefulness in MS
INFLAMMATION			
Cytokines			
Osteopontin	Macrophages	Mediate pro- and anti-inflammatory activity in the CNS. Proinflammatory cytokines significantly associated with disease activity.	Biomarkers of inflammatory disease activity. Not specific for MS.
TNF-α	CD4+ T cells		
MIF	Macrophages T cells		
IL-17	Th17 cells		
IL-4	Th2		
IL-6	Th2		
IL-10	Th2 Treg		
Chemokines and their receptors			
CXCL13	T cells, DC	Mediate immune cell recruitment into the CNS.	Biomarkers of inflammatory disease activity. Not specific for MS.
CXCL10	Monocytes Endothelial cells		
CCL2	Astrocytes Glial cells		
Antibodies and complement			
Anti-MOG	B cells	Reflect B cell mediated responses towards neuronal proteins	Biomarkers of disease activity and disability
Anti-MBP	B cells		
Anti-NF	B cells		
Apoptosis-related molecules			
Fas	T cells Oligodendrocytes	Mediate apoptotic events in immune and neuronal cells.	Biomarkers of inflammatory activity and disability progression
FasL	T cells		
TRAIL	T cells NK cells		
FLIP	All		
BIRC	All		
Bcl-2	All		

BBB FUNCTION			
Adhesion molecules			
sICAM	Endothelial cells	Regulate immune cell transmigration into the CNS	Biomarker for disease activity
sVCAM	Endothelial cells		
sP-selectin	Endothelial cells		
sE-selectin	Endothelial cells		
Matrix metalloproteinases			
MMP-2	MΦ T cells	Mediate BBB disruption and may be also responsible of degenerative events	Biomarkers of disease activity
MMP-9	MΦ T cells		
DEGENERATION			
Axonal and neuronal damage			
Neurofilament	Neuronal	Represent degenerative phenomena in MS	Biomarkers for disability
Tau	Neuronal		
14-3-3	Neuronal		
MBP	Neuronal		
Oxidative stress and excitoxicity			
NO	Macrophages Mast cells Astrocytes	Represent degenerative phenomena in MS	Biomarkers for disease activity and disability
NOS	Macrophages Mast cells		
Nitrate	Macrophages Mast cells		
Nitrite	Macrophages Mast cells		
REGENERATION			
NCAM	Immune cells	Neurotrophic factors in inflammatory lesions, which can stimulate regeneration and promote repair in the MS lesions	Disability markers
CNTF	Immune cells		
BDNF	Immune cells		
NGF	Immune cells		
NT-3	Immune cells		

Abbreviations: TNF tumor necrosis factor; MIF macrophage migration inhibitory factor; IL interleukin; CXCL CXC motif chemokine; MOG myelin oligodendrocytes glycoprotein; MBP myelin basic protein; NF neurofilament; TRAIL TNF-related apoptosis inducing ligand; FLIP FLICE-like inhibitory protein; BIRC baculoviral IAP repeat-containing; Bcl-2 B-cell lymphoma 2; ICAM Inter-cellular adhesion molecule; VCAM Vascular cell adhesion protein; MMP matrix metalloproteinase; NO nitric oxide; NOS NO synthetase; NCAM Neural cell adhesion molecule; CNTF ciliary neurotrophic factor; BDNF brain-derived neurotrophic factor; NGF nerve growth factor; NT-3 Neurotrophin-3; Th T helper cell; Treg Regulatory T cell; DC dendritic cell.

3.5 Magnetic resonance imaging as a surrogate marker for multiple sclerosis

MRI is an important tool in evaluating MS patients and it is by far the most extensively measured biomarker in MS. It has a high sensitivity for detecting focal abnormalities in the CNS. The changes seen by MRI in MS patients reflect underlying pathology: inflammation and axonal degeneration (Katz et al., 1993; Bruck et al., 1997). Typical magnetic resonance sequences include T1-weighted with or without Gadolinium (Gd) administration, T2 weighted, proton-density, and fluid-attenuated inversion recovery (FLAIR). The inflammatory disease process in MS is thought to be reflected by the detection of Gd-contrast enhancing T1 lesions and new T2 white matter lesions and the axonal loss by T1 hypointense lesions. Gd-enhanced T1-weighted images reflect increased BBB permeability associated with acute inflammatory activity of MS (Kermode et al., 1988; McFarland et al., 2002; Tomassini and Palace, 2009).

95% of patients with clinically definite MS have white matter lesion seen on brain MRI and their presence in patients with CIS is the strongest predictor of MS. The number and volumes of lesions correlated only weakly with future disability. Moreover, a determination of brain volumes has allowed an indirect quantification of cerebral tissue loss (brain atrophy) in MS patients and has been to shown to correlate with the disability. (Brex et al., 2002; McFarland et al., 2002)

In addition to conventional MRI (dual-echo, FLAIR, and T1-weighted imaging with and without Gd administration), newer MRI techniques, like diffusion tensor imaging (DTI) may add new information on aspects of the pathomechanism. These techniques have the great advantage of being more specific towards the heterogeneous pathological substrates of the disease than conventional MRI. In addition, they also allow quantifying and monitoring the extent of damage not only in lesions but also in normal-appearing white matter and grey matter. Finally, the development of new analysis approaches to assess functional MRI (fMRI) data is disclosing, at an unprecedented pace, the mechanisms of cortical reorganization following the accrual of tissue damage, which have the potential to limit its clinical consequences (Filippi and Agosta, 2010).

AIMS OF THE STUDY

MS is characterized by a variable clinical course and a heterogeneous and complex pathology and pathogenesis. Identification of immunological subtypes of MS in individual patients is an important issue. That will allow more focused application of different therapies in the management of MS. The goal of this thesis was to identify biomarkers in the blood that could reflect pathogenetic processes in MS and be used as biomarkers of disease activity and progression.

Our specific aims were to:

1) Define immune profiles in different clinical subtypes of MS and CIS
2) Search for biomarkers for disease activity and disability
3) Search for prognostic biomarkers for conversion of CIS to MS
4) Explore whether the postpartum disease activation of MS is related to changes in the apoptotic molecules in blood.

SUBJECTS AND METHODS

1. Subjects and ethical considerations (Studies I-IV)

The study included 232 subjects: 93 RRMS, 29 SPMS, 21 PPMS 18 CIS and 71 healthy controls (Table 6). The diagnosis of MS was based on McDonald's revised criteria and all diagnoses were definite (McDonald et al., 2001; Polman et al., 2005). CIS patients were defined as patients who had a first clinical episode suggestive of MS. All the patients were studied neurologically and blood samples were collected. The majority of the patients were studied during a remission (**studies I-IV**) and 25/93 of RRMS and 7/29 of SPMS patients were studied during relapse (**study I**). None of the patients had received methyl prednisolon during the eight weeks prior to study entry. 43/93 of RRMS patients were treated with IFN-β and 6/93 with GA (table 6). The primary aim of these studies was not to evaluate the clinical efficiency of IFN-β treatment and therefore the timing of IFN-β injection prior to blood sampling was not recorded. Some of the MS patients were studied neurologically and by MRI one year after their baseline visit (n=89, **study II**), as well as two years after their baseline visit (n=21, **study III**).

Clinical disease progression was evaluated by the Expanded Disability Status Scale (EDSS) (Kurtzke, 1983) at the baseline or changes in the EDSS score during the follow-up. Paraclinical disease progression was evaluated by volumes of T1 or changes in the volumes of T1 in the follow-up. Clinical disease activity was determined by calculating number of relapses proceeding two years (**studies I-III**) and paraclinical disease activity by the detection of Gd-contrast enhancing T1 lesions and volumes of FLAIR lesions by MRI (**Studies II and III**).

The study was approved by Ethics Committee of Tampere University Hospital and all subjects gave written informed consent.

Table 6. Clinical characteristics of all patients

	RRMS	SPMS	PPMS	CIS	All patients	CTRL	Total
N	93	29	21	18	161	71	232
Age[a]	36±11	48±9	57±9	34±9	40±13	37±12	39±13
F/M[b]	75/18	19/10	12/9	15/3	121/40	54/17	175/57
Duration,[a,c]	5.1±4.6	9.9±8.0	11.9±8.4	1.9±1.9	7.1±6.6	-	-
Relapses [a,d]	0.9±0.6[e]	1.8±1.4	0.7±1.2	-	1.4±1.4	-	-
EDSS[a]	1.7±1.6	4.6±1.8	4.8±2.0	0.1±0.2	2.5±2.3	-	-
Therapy[b]							-
NT	44	25	21	18	108	-	-
IFN	43	4	0	0	47	-	-
GA	6	0	0	0	6	-	-

Abbreviations: RRMS relapsing-remitting multiple sclerosis; SPMS secondary progressive multiple sclerosis; PPMS primary progressive multiple sclerosis. CIS clinically isolated syndrome; CTRL controls; EDSS expanded disability status scale; NT no treatment; IFN interferon-β; GA glatiramer acetate.

a Mean ± SD
b Number of patients
c Disease duration determined from the time of diagnosis for RRMS, SPMS and PPMS and from the first symptoms for CIS
d All relapses during preceding two year
e Disease activity from the **studies I-III**, pregnancy patients (**study IV**) disease activity were 4.1±3.1(mean±SD) and were determined by total number of relapses experienced before the onset of the study.

2. Molecular biology methods

2.1 Blood sampling and RNA isolation from peripheral blood mononuclear cells (Studies I-IV)

PBMC were separated in a VACUTAINER CPT cell preparation tube (Becton Dickinson and Company, Franklin Lakes, N.J., USA) according to the manufacturer's protocol. Total cellular RNA was isolated from stored cell lysate with Qiagen's RNase minikit (QIAGEN GmbH, Hilden, Germany) according to the

manufacturer's instructions. DNA was removed with Qiagen's RNase-Free DNase set. Total RNA was eluted to nuclease-free water and samples were stored -70 ºC. Sera were separated from blood and stored at -70 ºC.

2.2 Gene expression analysis by Lightcycler (Study I)

The expression of TRAIL mRNA could be determined in 46 patients and 17 controls in whom RNA was available. A relative quantitative real time polymerase chain reaction (RT-PCR) was performed with a LightCycler instrument (Roche Diagnostics GmbH, Mannheim, Germany). 1 µg of total RNA was reverse transcribed to complementary DNA (cDNA) with a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) following standard protocol with random hexamer primers.

Table 7. TRAIL primers and probes

Primer/Probe	Sequence
Forward primer	5'-AGAGAGTATGAACAGCCCCTG-3'
Reverse primer	5'-GTCCCAGTTATGTGAGCTGCTA-3'
Probe 1	5'-Red640-TTCTAACGAGCTGACGGAGTTGCCA-3'
Probe 2	5'- GGTTTCCTCAAGAGGTTCTCAAAAATCATC-FL-3'

Abbreviations: A adenine; C cytosine; G guanine; T thymine.

2-µl cDNA was used in a total of 20 µl reaction volume for the PCR reactions. The final concentrations of the target reagents in the system were: 1 x LightCycler FastStart DNA Master HybProbe, 4 mM MgCl2, 0,5 µM each primer and 0,2 µM each probe. PCR cycling conditions were: denaturation at 95 ºC for 10 min, amplification 45 cycles, at 95º C for 15 seconds, at 55 ºC for 15 seconds and at 72 ºC for 15 seconds and cooling at 40 ºC for 30 seconds. Primers and probes for the LightCycler PCR were designed and prepared by TIB MolBiol (Berlin, Germany) (Table 7). As a reference gene we used human glucose-6-phosphate-dehydrogenase (h-G6PDH). Reference gene PCR was done at the same time and the same PCR conditions as the target gene by a LightCycler® h-G6PDH housekeeping gene set kit (Roche Diagnostic GmbH, Mannheim, Germany). The reference gene was selected by a LightCycler h-Housekeeping Gene Selection Set, which included five alternative reference genes. All reactions for target and reference genes were made as

duplicate. Quantitative RT-PCR results were calculated by a LightCycler Relative Quantification Software with efficiency correction (Roche Diagnostics GmbH).

2.3 QRT-PCR using low density array (Study III)

Total-RNA (1 µg) was reverse transcribed to cDNA using High capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) with standard protocol. Gene expression analyses were done with TaqMan low-density array (LDA) (Applied Biosystems) using 7900HT Real-time PCR system (Applied Biosystems). Human apoptosis array was used to determine expression of 93 apoptotic-molecules and three housekeeping genes from PBMC. Arrays were loaded with 4 µl undiluted cDNA, 42 µl H_2O, and 50 µl PCR Universal master mix and run according to the manufacturer's instructions. Samples were run as duplicate.
The expression data was analyzed with RQ manager software using comparative Ct method (ΔΔCt). To normalize the results, the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used and two healthy control samples were used as calibrator in the data analysis.

2.4 Flow cytometry analysis (Study I)

PBMC for TRAIL membrane expression analyses was obtained from 9 RRMS patients and 6 healthy controls. PBMCs (2 x10 5) were incubated with mouse anti-human CD3-APC-Cy7, CD4-PE-Cy7, CD8-APC, CD14-FITC and TRAIL-PE (Becton Dickinson, Franklin Lakes, NJ, USA) monoclonal antibodies for 20 min. As an isotype control, cells were incubated with mouse anti-human IgG1-APC-Cy7, IgG1-PE-Cy7, IgG1-APC, IgG1-FITC and IgG1-PE monoclonal antibodies. Thereafter cells were washed twice, resuspended in PBS, and analyzed with FACSAria flow cytometry and Facsdiva software (Becton Dickinson). Stainings were made in duplicate. Optimization of fluorescence compensation was made using BD CompBeads (Becton Dickinson).

2.5 Enzyme-linked immunosorbent assay (Studies I, II and IV)

Levels of sTRAIL in undiluted sera were quantified by a Diaclone solid phase sandwich ELISA kit (# 850.770.096; Diaclone, Besancon Cedex, France). Detection limits were 64 pg/ml. TRAIL absorbancies were read with a Multiskan MS version 4.0 spectrophotometer (Labsystems, Helsinki, Finland) at wavelength 450 nm.

2.6 Luminex (Studies I, II and IV)

The collected blood was allowed to clot for at least 30 minutes before separation. The blood containing tubes were then centrifuged for 15 minutes at 1600 x g. Sera were separated from the blood, aliquoted and stored at -70 °C.

The levels of sFas, sFasL and MIF were measured with a Human sepsis/apoptosis LINCOplex Kit (Linco Research, St. Charles, Missouri, USA) **(Studies I, II, and IV)**, CCL2, CCL3, CCL4 and CXCL10 with a Human cytokine LINCOplex kit (Linco Research) **(Study II)** and IL-2, IL-6, IL-10, IL-12p70, IFN-γ and TNF-α with High Sensitivity Human cytokine LINCOplex kit (Linco Research) **(Study II)**. All the data were collected and analyzed using Bio-Plex suspension array system and Bio-Plex Manager software 4.1 (Bio-Rad laboratories, California, USA). A five-parameter regression formula was used to calculate the sample concentration from the Human sepsis/apoptosis LINCOplex Kit and the High Sensitivity Human cytokine LINCOplex kit, and a four-parameter regression formula was used for the Human cytokine LINCOplex kit. All 96 well plates included samples from all disease subtypes and controls to minimize inter-assay variation. The same batch of monoclonal antibodies for the Bio-Plex Cytokine Assay System was used throughout the experiments; the inter-assay and intra-assay values are reported to be less than 15% by the manufacturer. The percent recovery of standards ranged from the 90% to 110% that was used as a detection limit for each protein. The lower detection limits were as follows: 12.2 pg/ml for sFas, sFasL and MIF, 16.0 pg/ml for CXCL10, CCL3 and CCL4, 3.2 pg/ml for CCL2, 0.13 pg/ml for IL-10, TNF-a, IL-6, IL-12p70, IL-2 and IFN-g.

3. Magnetic resonance imaging (Studies II and IV)

All examinations were performed on a 1.5 Tesla MRI Unit (Siemens Avanto, Erlangen, Germany). The MRI protocol for this examination included a T1 weighted header followed by an axial T1- weighted magnetization prepared rapid gradient echo (MP-RAGE), and a T2-weighted turbo spin-echo (TSE), fluid attenuation inversion recovery (FLAIR), magnetization transfer contrasts (MTC), diffusion weighted imaging (DWI), and gadolinium enhanced T1 weighted MP-RAGE sequences. In this study, T1 weighted MP-RAGE, FLAIR and T2-weighted TSE images were used for volumetric analysis. For MP-RAGE, the imaging parameters were as follows: repetition time (TR) = 1160 ms; echo time (TE) = 4.24 ms; inversion time (TI) = 600 ms; slice thickness=0.9 mm; in-plane resolution = 0.45*0.45 mm. In FLAIR, the following parameters were used: TR = 8500 ms; TE = 100 ms; TI = 2500 ms; slice thickness= 5.0 mm; in-plane resolution = 0.45*0.45 mm. In TSE, the following imaging scheme was used: TR = 750 ms; TE = 115 ms; slice thickness= 3.0 mm; in-plane resolution = 0.90*0.90 mm. Volumetric segmentation of plaques in the brain was performed using semiautomatic software Anatomatic™ operating in a PC/Window 95 environment (Heinonen et al., 1998a; Heinonen et al., 1998b) and the images were analyzed blindly.

4. Cluster analysis (Study III)

The gene expression patterns were used to cluster the samples and genes by two-way hierarchical clustering. Absolute expression change values ≥1.5 and ≤0.67 were used as cut-offs to define differentially expressed genes. Genes that showed ≥1.5 and ≤0.67-fold change in their expression level compared to control were regarded to be upregulated and downregulated, respectively. Hierarchical clustering was applied to both axes with the Cluster program (Michael Eisen,

http://rana.lbl.gov/EisenSoftware. htm) using uncentered correlation as a similarity metric. Results were visualized with the TreeView program (Michael Eisen, http://rana.lbl.gov/EisenSoftware.htm).

5. Statistical analyses (Studies I-IV)

Statistical analyses were performed using SPSS version 14.0 (**studies I-II**) and 16.0 (**studies III-IV**) for Windows (SPSS Inc., Chicago, Illinois, USA). Results of gene expression were calculated by using the independent samples T-test (**study I**) and the Mann–Whitney U test (**Study IV**). A p values less than 0.05 were considered statistically significant. Results of serum cytokines were tested by non-parametric analyses: the Kruskal–Wallis test for comparison of more than two unpaired groups (**study III**), the Mann–Whitney U Test for comparison of two unpaired groups (**studies I-III**), and the Wilcoxon test for paired groups (**studies II-III**). A p values less than 0.05 (**studies I-II**) or 0.01 (**studies III**) were considered statistically significant. Correlations between variables were tested by two-tailed non-parametric Spearman correlation analysis. Due to multiple correlation testing, a p values of less than 0.01 were considered significant. Correlations between variables were tested by two-tailed non-parametric Spearman correlation analysis and a p values of less than 0.01 were considered significant (**studies I-IV**).

RESULTS

1. Candidate immunological biomarkers (Studies I-III)

1.1 Characterization of immune profiles in different subtypes of multiple sclerosis

We characterized serum profiles of apoptosis-related molecules (sFas, sFasL, sTRAIL, TNF-α), chemokines (CCL2, CCL3, CCL4, CXCL10) and cytokines (MIF, IL-2, IL-6, IL-10, IL-12p70, IFN-γ) in all the MS subtypes during the disease stable phase **(Studies I and II)**. These molecules are considered to be important in the MS pathogenesis. Elevated serum levels of sFas, TNF-α and CCL2 in PPMS and decreased levels of MIF were found in RRMS when compared to CIS, controls and other MS subtypes (**Study II**, Table 8). The levels of apoptosis-related molecules sFasL, sTRAIL, chemokines CCL3, CCL4, CXCL10 or cytokines IL-2, IL-10, IL-6, IL-12p70, IFN-γ did not differ statistically between the groups ($p>0.01$).

In addition to the soluble form of the TRAIL, we also studied the membrane bound form of the TRAIL in peripheral blood CD4+ and CD8+ T cells and CD14+ monocytes. No differences in the surface expression of TRAIL on CD4+ and CD8+ T cells and CD14+ monocytes was found in the 9 MS patients and 6 healthy controls. The surface expression of TRAIL was detectable in less than 1 % of CD3+CD4+ or CD3+CD8+ T cells and in less than 5 % of monocytes.

Table 8. The levels of soluble molecules in different types of MS and CIS and controls. Median (25th-75th percentiles) (Study II)

Molecule	CDMS N=72	RRMS N=33	SPMS N=18	PPMS N=21	CIS N=17	HC N=21
Fas	5394.3 (4343.7–6833.5)	4612.8 [d] (4058.0–5940.7)	5572.4 (4388.3–6845.4)	6787.6 [c,d] (5393.9–7709.2)	4318.8 (3320.0–5697.9)	4824.9 [c] (3405.1–5638.1)
FasL	94.1 (67.6–168.6)	90.6 (58.1–161.6)	96.2 (72.8–153.6)	111.2 (71.5–191.9)	83.6 (67.1–174.6)	80.4 (56.6–129.1)
MIF	195.0 [a] (100.2–298.0)	138.2 [b,d] (50.0–229.4)	225.8 (134.0–317.3)	290.7 [d] (189.3–449.3)	167.3 (97.8–268.2)	378.5 [a,b] (236.4–535.9)
TRAIL	845.2 (645.2–1603.0)	765.9 (577.2–1728.9)	785.5 (587.2–1106.0)	1032.0 (762.8–2036.1)	902.3 (593.3–1170.5)	738.7 (523.2–2260.8)
CXCL10	98.9 (66.1–154.7)	95.7 (68.1–155.6)	90.5 (62.8–152.6)	106.9 (74.7–212.9)	65.9 (53.6–178.2)	106.9 (55.1–130.7)
CCL2	218.3 (154.7–371.4)	177.0 (140.4–271.8)	218.8 (157.0–331.0)	288.4 [c] (201.3–461.6)	248.3 (165.7–332.0)	152.8 [c] (122.6–273.1)
CCL3	50.9 (22.4–99.3)	37.3 (21.6–96.3)	55.7 (19.2–121.4)	45.4 (27.0–164.7)	72.6 (32.0–186.1)	51.4 (27.7–122.8)
CCL4	160.6 (24.6–835.5)	126.6 (20.4–668.0)	140.2 (17.0–873.8)	290.7 (39.8–1054.6)	365.1 (144.0–1188.5)	289.1 (16.8–725.0)

IL-10	3.2 (1.6–7.1)	2.9 (0.6–5.7)	3.4 (1.8–7.7)	3.6 (2.2–9.2)	4.8 (1.2–25.9)	2.3 (0.2–6.0)
TNF-α	4.7 [a] (3.7–7.3)	4.5 [d] (3.4–6.5)	4.1 (3.3–5.8)	6.4 [c,d] (4.4–8.9)	4.6 (3.5–6.6)	3.7 [a,c] (2.4–4.6)
IL-6	7.0 (2.1–18.4)	4.9 (2.0–21.5)	7.1 (3.2–13.0)	8.8 (1.6–26.7)	3.6 (1.4–17.9)	4.1 (2.0–9.3)
IL-12p70	0.6 (0.1–3.0)	0.4 (0.1–1.4)	1.3 (0.4–5.4)	0.8 (0.1–4.5)	1.7 (0.3–9.1)	1.6 (0.1–7.9)
IFN-γ	0.6 (0.3–3.5)	0.3 (0.3–3.0)	1.5 (0.3–4.1)	0.6 (0.3–3.3)	2.8 (0.3–25.5)	0.5 (0.3–7.5)
IL-2	0.6 (0.2–3.6)	1.2 (0.2–3.6)	0.6 (0.2–5.2)	0.4 (0.2–2.4)	1.5 (0.5–6.2)	2.1 (0.2–6.1)

Abbreviations: CDMS clinically definite MS (RRMS, SPMS and PPMS); RRMS relapsing-remitting MS; SPMS secondary progressive MS; PPMS primary progressive MS; CIS clinically isolated syndrome; HC healthy controls; Fas L Fas ligand; MIF macrophage migration inhibitory factor; TRAIL tumor necrosis factor -related apoptosis inducing ligand; CXCL C-X-C motif chemokine ligand; CCL C-C motif chemokine ligand; IL interleukin; TNF tumor necrosis factor.

a Comparison between CDMS and HC group, the Mann-Whitney U Test $p<0.01$

b Comparison between RRMS and HC group, the Mann-Whitney U Test $p<0.01$

c Comparison between PPMS and HC group, the Mann-Whitney U Test $p<0.01$

d Comparison between the PPMS and RRMS group, the Mann-Whitney U Test $p<0.01$

Table 9. Significantly upregulated genes (fold change ≥ 1.50 and p≤0.01) in CIS, RRMS and controls.

Gene	Gene expression values[a]			Fold change[b]		p-value[c]	
	RRMS N=11	CIS N=10	Control N=7	RRMS	CIS	RRMS	CIS
BAD	1.38 (1.00–3.85)	1.06 (0.93–1.15)	0.84 (0.75–1.00)	1.64*	1.26	0.01	0.02
BBC3	1.32 (0.91–2.73)	1.03 (0.82–1.23)	0.80 (0.52–1.00)	1.65*	1.29	0.01	0.09
BCL2L14	1.72 (1.34–2.15)	1.38 (1.04–2.62)	1.00 (0.34–1.18)	1.72*	1.38	0.01	0.03
BNIP3	1.36 (1.16–1.56)	1.69 (1.30–1.84)	1.00 (0.72–1.05)	1.36	1.69*	0.05	0.01
TNFRSF25	2.73 (1.81–3.85)	3.05 (2.16–3.36)	1.16 (1.00–1.49)	2.35*	2.63*	0.01	0.00
IKBKE	1.50 (1.05–1.90)	1.55 (1.33–1.62)	1.00 (0.95–1.14)	1.50*	1.55*	0.01	0.01
NFKBID	1.76 (1.36–2.05)	1.77 (1.54–1.97)	1.00 (0.92–1.30)	1.76*	1.77	0.01	0.03

Abbreviations: RRMS relapsing-remitting multiple sclerosis; CIS clinically isolated syndrome; BAD BCL2-associated agonist of cell death; BBC3 BCL2 binding component 3; BCL2L14 Apoptosis facilitator Bcl-2-like protein 14; BNIP3 BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; TNFRSF25 tumor necrosis factor receptor superfamily member 25; IKBKE inhibitor of nuclear factor kappa-B kinase subunit epsilon; NFKBID nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor.

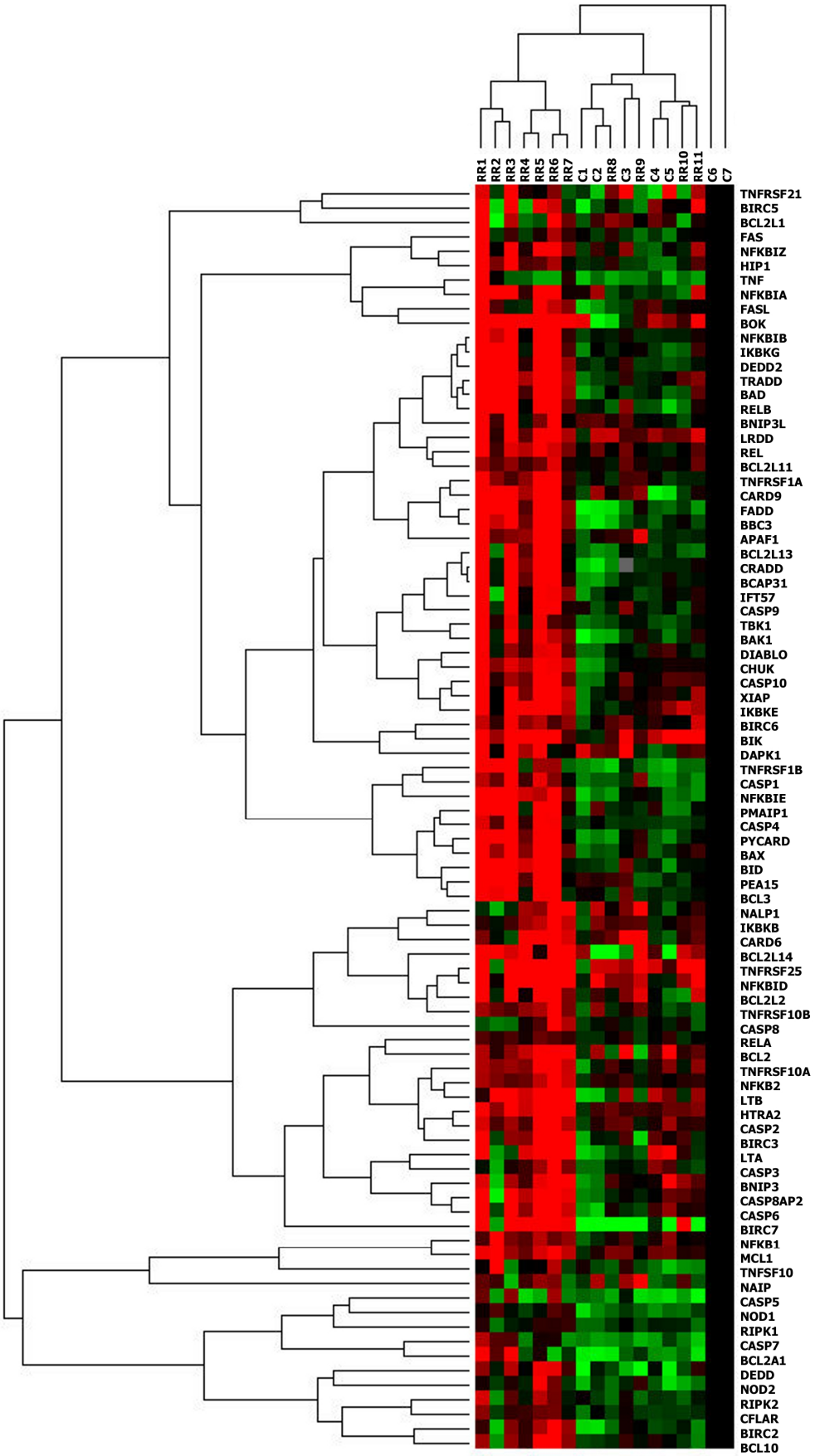
a The expression values are medians and numbers in parentheses interquartile ranges (q1–q3).

b Fold changes are calculated by comparing median gene expression values between patients and control.

c P-values between MS subtype and controls from Mann-Whitney U test.

Gene expression profiles of apoptosis-related genes in early MS patients were also examined (**Study III)**. In RRMS, six genes including the Bcl-2 family *(BAD, BBC3, BCL2L14)*, the death receptor pathway *(TNFRSF25)* and NF-κB family *(IKBKE, NFKBID)* were upregulated when compared to controls (Table 9). In CIS patients, three genes *BNIP3, TNFRSF25* and *IKBKE* appeared to be upregulated in comparison to controls. Thereafter, expression of all the genes in RRMS, CIS, and controls were compared with cluster analysis (Figure 8). In RRMS, almost all of the genes were upregulated when compared to controls (Figure 8A). Comparison between CIS and controls showed upregulated several genes in two regions in the dendrogram in CIS (Figure 8B). These genes are listed in Table 3 in **study III**.

A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C1
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C6
C7
TNFRSF21
BIRC5
BCL2L1
FAS
NFKBIZ
HIP1
TNF
NFKBIA
FASL
BOK
NFKBIB
IKBKG
DEDD2
TRADD
BAD
RELB
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LRDD
REL
BCL2L11
TNFRSF1A
CARD9
FADD
BBC3
APAF1
BCL2L13
CRADD
BCAP31
IFT57
CASP9
TBK1
BAK1
DIABLO
CHUK
CASP10
XIAP
IKBKE
BIRC6
BIK
DAPK1
TNFRSF1B
CASP1
NFKBIE
PMAIP1
CASP4
PYCARD
BAX
BID
PEA15
BCL3
NALP1
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BCL2L14
TNFRSF25
NFKBID
BCL2L2
TNFRSF10B
CASP8
RELA
BCL2
TNFRSF10A
NFKB2
LTB
HTRA2
CASP2
BIRC3
LTA
CASP3
BNIP3
CASP8AP2
CASP6
BIRC7
NFKB1
MCL1
TNFSF10
NAIP
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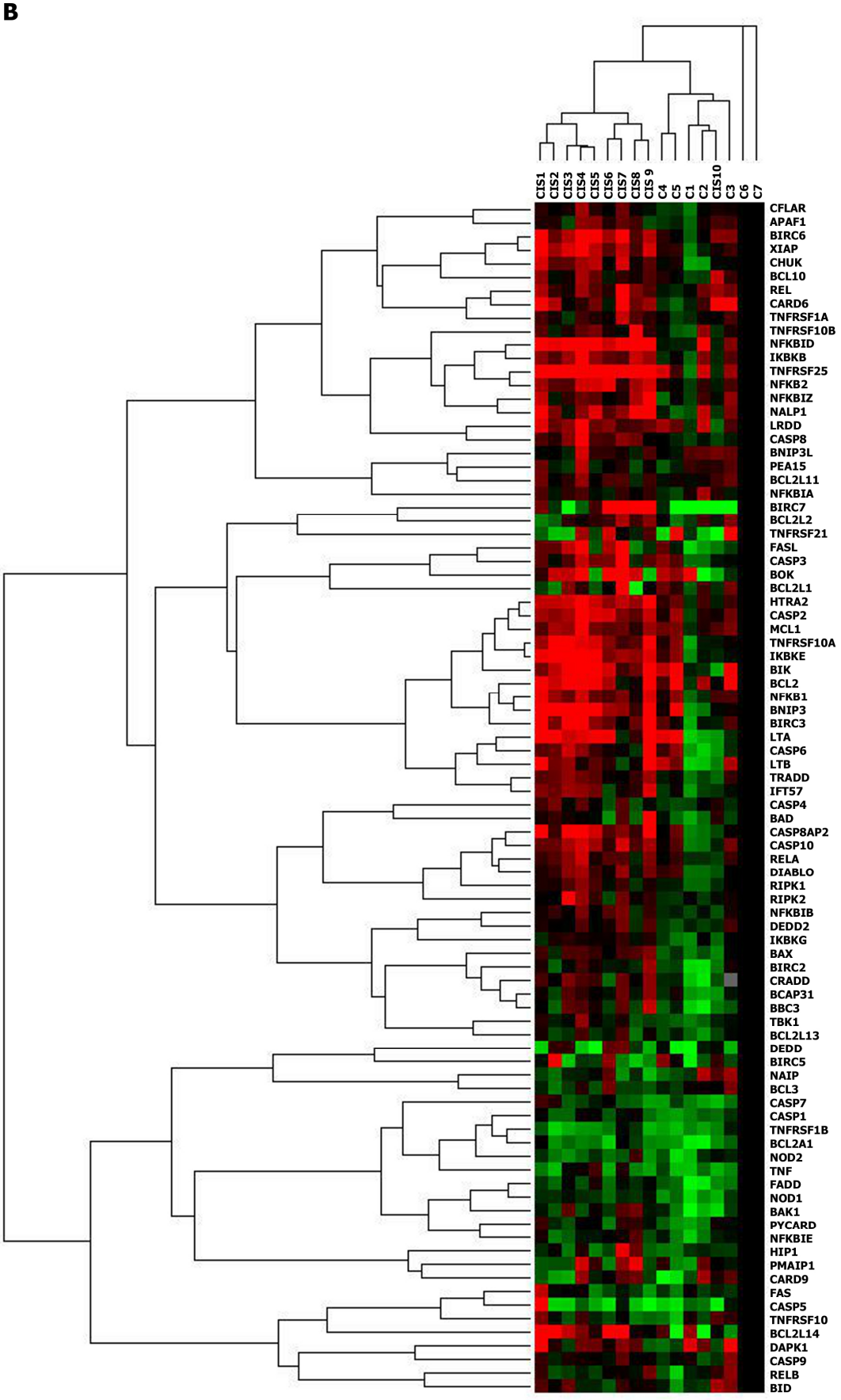
B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C7
CFLAR
APAF1
BIRC6
XIAP
CHUK
BCL10
REL
CARD6
TNFRSF1A
TNFRSF10B
NFKBID
IKBKB
TNFRSF25
NFKB2
NFKBIZ
NALP1
LRDD
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BNIP3L
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NFKBIA
BIRC7
BCL2L2
TNFRSF21
FASL
CASP3
BOK
BCL2L1
HTRA2
CASP2
MCL1
TNFRSF10A
IKBKE
BIK
BCL2
NFKB1
BNIP3
BIRC3
LTA
CASP6
LTB
TRADD
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CASP4
BAD
CASP8AP2
CASP10
RELA
DIABLO
RIPK1
RIPK2
NFKBIB
DEDD2
IKBKG
BAX
BIRC2
CRADD
BCAP31
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NFKBIE
HIP1
PMAIP1
CARD9
FAS
CASP5
TNFRSF10
BCL2L14
DAPK1
CASP9
RELB
BID

Figure 8. **Hierarchical clustering showing relative gene expression comparing patients with RRMS (A) and CIS (B) to healthy controls.** The gene expression patterns were used to cluster the samples and genes by two-way hierarchical clustering. Absolute expression change values ≥1.5 and ≤0.67 were used as cut-offs to define differentially expressed genes. Genes that showed ≥1.5 and ≤0.67-fold change in their expression level compared to control were regarded to be upregulated (red) and downregulated (green), respectively.

1.2 Immunological biomarkers in relation to the clinical and subclinical multiple sclerosis disease activity and disability

1.2.1 Candidate biomarkers of disease activity

Biomarkers association to disease activity of RRMS and SPMS patients can be studied by several methods. In this study, clinical disease activity was determined by calculating number of relapses in the preceding two years (**studies I-III**) and paraclinical disease activity by the detection of Gd-contrast enhancing T1 lesions and volumes of FLAIR lesions by MRI (**Studies II and III**). In addition, candidate biomarkers associations to disease activity were also studied in samples that were collected during clinical relapse (**Study I**).

Most of the samples and MRI scans in this study were collected during the disease stable phase. Correlation analyses between clinical disease activity and analyzed molecules did not reveal significant results. Also no associations were found between the volumes of FLAIR white matter lesions and soluble molecules in the whole MS group. However, in the combined group of patients with RRMS and SPMS, significant correlation was detected between the levels of IL-6 and the volumes of FLAIR lesions ($r=0.368$ $p=0.009$, $n=51$, **study II**).

During relapse, the levels of sTRAIL and MIF were higher in untreated patients with relapse when compared to controls or untreated patients in remission (**Study I**, Figure 9). The levels of sFas and sFasL were without significant differences between these comparisons.

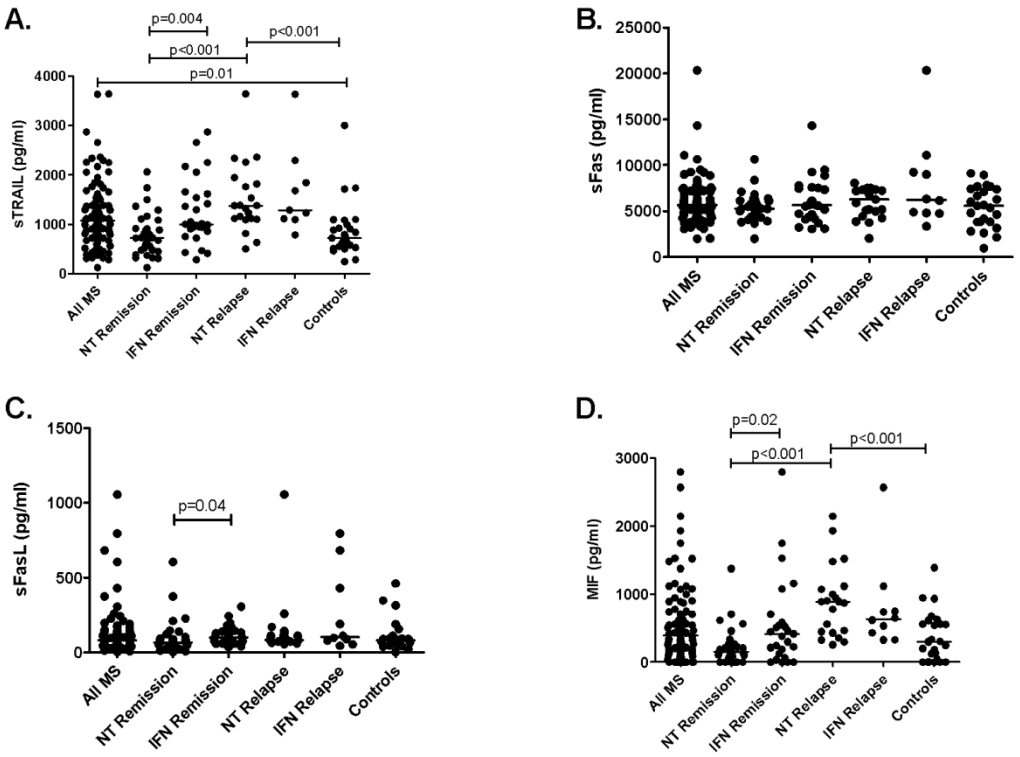

Figure 9. **The median levels of sTRAIL (A), sFas(B), sFasL (C) and MIF (D) in sera of all MS patients (n=91), untreated MS patients during remission (n=32), IFN-β treated MS patients during remission (n=27), untreated MS patients during relapse (n=22), IFN-β treated MS patients during relapse (n=10) and healthy controls (n=26).** The levels of cytokines are shown for each donor. IFN= interferon-β, NT= no treatment. Picture adopted from the article by Rinta et al., 2008, reproduced with permission of the publisher.

Correlation analyses between gene expression and clinical disease activity showed a positive correlation for *BCL2L1* (r=0.777, p=0.008) and *FAS* (r=0.763, p=0.010) in RRMS patients (**study III**). In addition, negative correlations were found between the volumes of FLAIR and nine of the apoptosis-related genes and four out of these nine genes correlated negatively with the volumes of T1 lesions.

1.2.2 Candidate biomarkers of disease progression

Disease progression was evaluated by EDSS score or the presence of new T1 changes. Therefore, correlation analyses between immunological molecules and EDSS scores, progression index and the volumes of T1 lesions were performed. The expression of TRAIL mRNA correlated positively with EDSS score (r=0.38,

spearman p=0.01) and especially in the IFN-β treated RRMS patients in remission (n=21) the expression of TRAIL mRNA correlated positively with both EDSS score (r=0.59, spearman p=0.005) and progression index (r=0.56, p=0.009) (**Study I**,). Also, positive correlations between serum MIF and the EDSS score in the CDMS group (including all subtypes) were found (r=0.39, p=0.001, n=72, Figure 10A), but within the subtypes statistically significant correlations were not found (**study II**).
At one-year follow up, EDSS score had increased by 31% of CDMS patients. Correlation analyses did not reveal significant changes between EDSS change and immunological findings. Therefore, to analyze the association between measured biomarkers and disease progression over the year, CDMS patients were divided into two groups: patients with disability progression (EDSS score increased more than 0.5, n=20) and those with stable EDSS score (EDSS score unchanged, n=44). In the worsening patients, the levels of sFas and MIF were higher than in the stable group (Figure 10 B and C).

Similarly, correlation analyses between T1 and FLAIR change and immunological molecules were not significant. To further evaluate the association between immunological responses and MRI changes consistent with neurodegeneration, the patients with RRMS and SPMS were divided into two groups: those with increased volumes of T1 over the year (progressed, $\Delta T1 \geq 1.0$ cm3, n=19) and stable ones (stable, $\Delta T1 < 1.0$, n=27). Upregulated levels of sFas were found in patients with increasing T1 lesion volumes in comparison with the stable group (Figure 10 D).

1.2.3 Conversion-related markers

Over the one-year follow up, three out of seventeen CIS patients converted to CDMS, but we could not associate any of the immune markers to the conversion (**study II**). At the two-year follow-up, five out of ten patients fulfilled the diagnostic criteria of MS and those converted patients showed upregulation of three genes (*APAF, BIRC6, CFLAR*) when compared to controls **(study III)**.

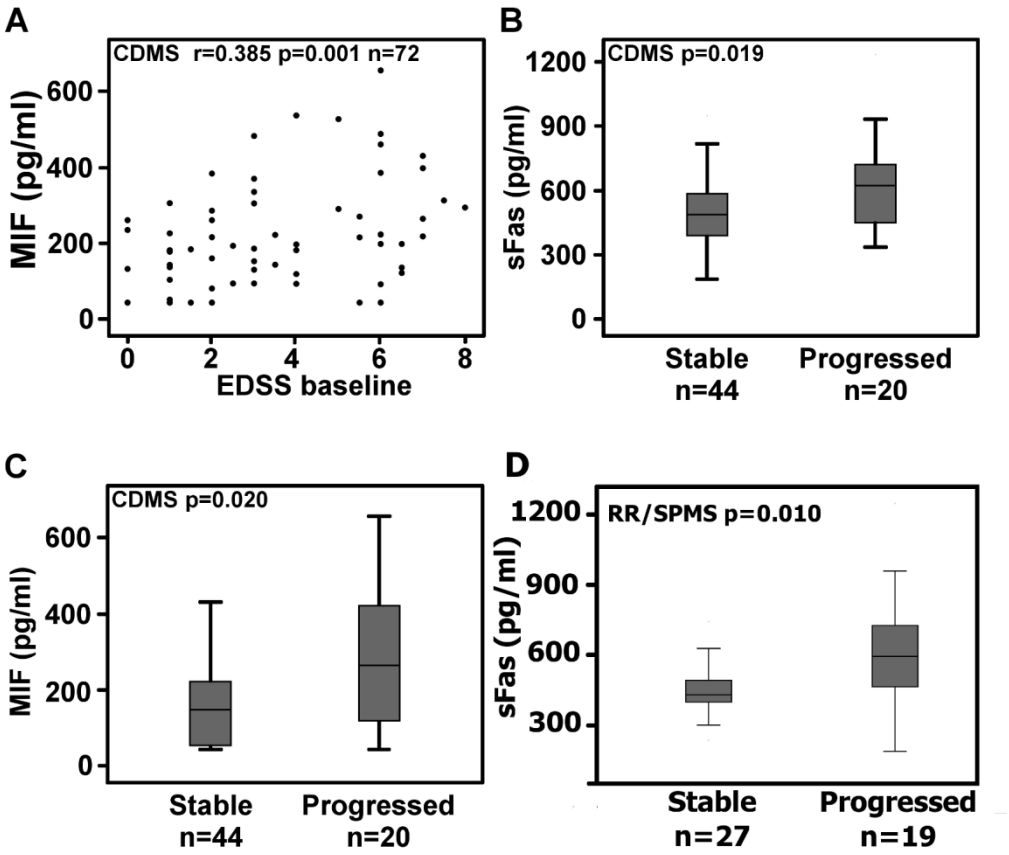

Figure 10. **Association the levels of MIF and sFas to disease progression.** **(A)** Correlations between baseline EDSS score and the levels of MIF in clinically definite MS (CDMS) group. **(B-C)** Increased levels of sFas and MIF were found in patients with disability progression in CDMS group. CDMS patients were divided into two groups: patients with disability progression (EDSS score increased more than 0.5, n=20) and those without progression of disability (EDSS score unchanged, n=44) over one year follow-up. **(D)** Upregulated levels of sFas were found in those RRMS and SPMS patient whose T1 lesion volumes increased in comparison to stable group of patients. RRMS and SPMS patients were divided in the two groups: those with increased volumes of T1 from baseline to one-year follow-up (progressed, n=19) and those with unchanged T1 volumes (stable, n=27).The length of the box represents the interquartile range within which 50% of the values were located. The line through the middle of each box represents the median. The error bars show the minimum and maximum values (range). Modified from Hagman et al., 2011.

2. Reconstitution of immune activity after pregnancy (Study IV)

The study was undertaken to understand whether the postpartum disease activation of MS is associated with quantitative changes of apoptotic molecules in serum. Samples were obtained during the late pregnancy and postpartum from 19 RRMS patients and 14 controls. The levels of sTRAIL and sFasL increased from the pre- to postpartum periods in both groups (Figure 11). Moreover, in both pro- and postpartum situations the levels of sTRAIL were lower in RRMS patients than in controls. Comparing the apoptosis-related molecules changes (Δ) from late pregnancy to postpartum showed smaller changes in the sTRAIL in MS patients than in controls.

No correlations were found between the levels of serum apoptotic molecules and annualized relapse rate or EDSS score, or changes (Δ) in the levels of apoptotic proteins with annualized relapse rate or EDSS score ($p>0.01$). In order to further explore the relationship between quantitative changes in the apoptotic molecules and activity of MS, the patients were divided into an active group (n=11, up to three relapses after pregnancy and a stable group (n=7, no relapses). Comparison between these groups showed that the patients with active MS had smaller ΔFasL than the patients with inactive disease.

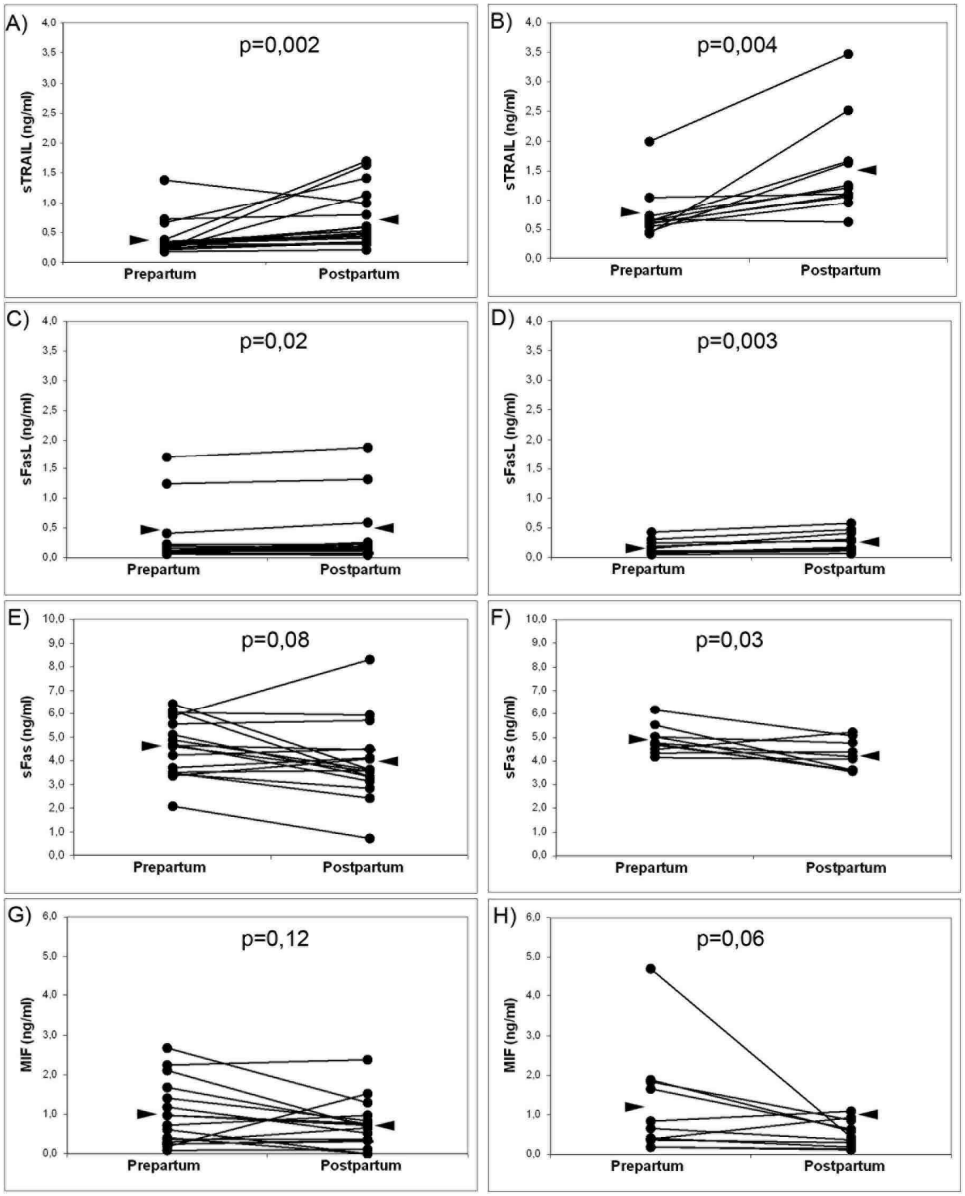

Figure 11. **The levels (ng/ml) of sTRAIL (A-B), sFasL (C-D), sFas (E-F) and MIF (G-H) during late pregnancy (prepartum) and after delivery (postpartum) in MS (A, C, E and G) and healthy controls (B, D, F and H).** The levels of molecules are shown for each donor. The arrowhead indicates the mean value. Picture adopted from the article by Rinta et al., 2010, reproduced with permission of the publisher.

DISCUSSION

1. Candidate immunological biomarkers (Studies I-III)

1.1 Characterization of immune profiles in different subtypes of multiple sclerosis and CIS

1.1.1 Primary progressive multiple sclerosis

In this thesis, we showed altered expression of immune-profiles in MS subtypes in both protein and gene expression levels. At the protein level, we observed a robust increase in sFas, TNF-α and CCL2 concentrations in PPMS patients that is consistent with the presence of inflammatory activity in this subtype. Previously, it was suggested that the PPMS subtype is a predominantly noninflammatory subtype, since new Gd-enhancing lesions are rare or absent and ongoing neurodegeneration is not correlated with the number or appearance of new focal white matter lesions (Bradl and Lassmann, 2009). Current DMT have little or no beneficial effect in the progressive stage of the disease. Therefore, it has been suggested that neurodegenerative events in the chronic progressive patients develop independently from inflammation. However, the most recent clinical trial on the efficacy of Rituximab in PPMS indicated delayed time to confirmed disease progression in Rituximab-treated patients with disease activity on MRI and this supports the concept of the inflammatory nature of PPMS patients (Hawker et al., 2009).

The involvement of the Fas-FasL system in the pathogenesis of MS has been suggested by several authors (Zipp, 2000; Pender, 2007), but the exact role of sFas and sFasL in pathogenesis of MS is still not known. Fas-FasL is one of the death receptor-ligand systems of T cells that are crucial in the apoptotic signaling pathway

(Askenasy et al., 2005). In particular, death receptor Fas defects have been reported in the T lymphocytes of MS patients, and especially in the chronic progressive subtype. These defects were considered to promote the survival of autoreactive T cells (Comi et al., 2000; Sharief, 2000a). The soluble forms of Fas have been shown to inhibit apoptotic events in T cells (Aktas et al., 2006). Our observation of increased sFas in PPMS is in line with previous data thus favoring the concept of dysregulated Fas-mediated apoptosis in MS (Ciusani et al., 1998; Boylan et al., 2001). In addition, TNF-α and CCL2 have been identified in active MS lesions (Selmaj et al., 1991) and increased levels of TNF-α in sera and CSF together with intrathecal synthesis of CCL2 have been reported in PPMS (Sharief and Hentges, 1991; Hohnoki et al., 1998). TNF-α is a well-known trigger of the apoptosis of oligodendrocytes (Watzlawik et al., 2010), while CCL2 is involved in the recruitment of monocytes and T cells from blood to brain parenchyma (Mahad and Ransohoff, 2003).

Taken together, our findings of increased levels of sFas, TNF-α and CCL2 in sera of PPMS patients together with earlier evidence are consistent with the involvement of these molecules in the pathogenesis of this subtype. This study also supports the hypothesis that the inflammation is present also in the PPMS subtype. Therefore, identification of immunological subtypes more carefully in this disease form may allow development of immunomodulatory therapies for PPMS patients.

1.1.2 Relapsing-remitting multiple sclerosis and clinically isolated syndrome

The gene expression analyses also showed aberrant immune profiles in RRMS and CIS patients. In RRMS, six proapoptotic genes that are members of the Bcl-2 and NF-Kβ families or included death receptor pathway were upregulated. Three of these genes are members of the Bcl-2 family, which are present on the mitochondrial membrane and include both pro- and antiapoptotic molecules. The balance between such pro- and antiapoptotic Bcl-2 family molecules determines the death or survival of cells (Brenner and Mak, 2009). Since one of the important roles of the immune system is to maintain peripheral homeostasis and thereby prevent inflammatory-driven pathogenetic events (Gregersen and Behrens, 2006), upregulation of

proapoptotic Bcl-2 family genes in RRMS indicates that the regulatory effects of the immune system are directed to the enhancement of apoptotic events in peripheral blood.

Furthermore, enhanced gene expression of *TNFRSF25* was found in both RRMS and CIS. TNFRSF25 is a receptor of the TNF-family that is constantly expressed in T lymphocytes, and its expression is strongly upregulated after T-cell activation (Croft, 2009). TNFRSF25 may either activate NF–κB and MAP-kinase signaling or alternatively trigger caspase activation and programmed cell death (Chinnaiyan et al., 1996; Wen et al., 2003).

Upregulated gene expression of IKK and NF-κB-related molecules was also found in RRMS and CIS. NF-κB related genes are essential in promoting proinflammatory events in immune cells (Krammer et al., 2007). IKBKE is the IKK-related kinase that has an essential role in innate immunity through activation of NF-κB, interferon regulatory factor (IRF)-3 and IRF7 (Hacker and Karin, 2006). The expression of BCL2 and BCL2L1 is also largely regulated by TCR mediated NF-κB activation.

In summary, aberrant gene expression profiles were seen in RRMS as well as CIS patients that suggest that apoptotic events are an important part of the MS pathogenesis. Upregulation of these pro- and anti-apoptosis molecules indicate the regulatory effect of the immune system in maintaining peripheral homeostasis and thereby preventing pathogenetic events.

1.2 Immunological biomarkers in relation to the multiple sclerosis clinical and subclinical disease activity and disability

1.2.1 Candidate biomarkers of disease activity

The primary aim in the field of biomarkers is to find sensitive, relatively inexpensive blood or CSF biomarkers that can detect inflammatory activity as well as the degree of neurodegeneration. MRI and OCB in CSF is the main paraclinical tool used in clinical practice (McFarland, 2009), but clinicoradiological correlations have been generally weak thus far (Barkhof, 2002).

Serum levels of MIF were upregulated during relapse and downregulated during remission and that may indicate the importance of this molecule as a disease activity marker. In line with our observation, Niino et al., have detected increased levels of MIF in CSF from relapsing multiple sclerosis patients (Niino et al., 2000). MIF is released from cytoplasmic stores following stimulation by endotoxin, exotoxin and cytokines such as TNF and IFN-γ and it can act in an auto- and paracrine fashion to stimulate its own synthesis and the synthesis of other proinflammatory mediators (Calandra et al., 1994). Studies using EAE have shown that MIF induces the accumulation of encephalitogenic T cells in the CNS thus facilitating the development of EAE (Denkinger et al., 2003; Kithcart et al., 2010). More recently, MIF was identified as a non-cognate ligand of the chemokine receptors CXCR2 and CXCR4 (Bernhagen et al., 2007). Taken together, these observations suggest that MIF is associated with disease activity and can strengthen the effects of other proinflammatory stimuli. Upregulated serum sTRAIL during relapse indicates that determination of this cytokine may be useful in the evaluation of MS disease activity. TRAIL is an anti-inflammatory molecule and upregulated TRAIL in sera may exert neuroprotective effects through inhibition of proinflammatory activity during relapse. However, this concept is in contrast with an observation of apoptosis-inducing effects of TRAIL in human brain reported by Nitsch et al. (Nitsch et al., 2000).

One tenth of the expression of apoptosis-related genes in this study correlated negatively with the volumes of FLAIR lesions. Among these molecules antiapoptotic BIRC2 and BIRC3 correlated with the volumes of FLAIR and T1 lesions, which is consistent with the presence of inflammatory activity in the brain. BIRC2 and BIRC3 are members of the inhibitor of apoptosis proteins (IAP) family that prevents apoptosis by interfering in the activation of caspases and ubiquitin-dependent signaling events that regulate activation of NF-κB transcription factor (Gyrd-Hansen and Meier, 2010). It has been reported that protein expression of BIRC2, BIRC3, XIAP, BIRC5 in mitogen-stimulated T cells from active RRMS patients has been elevated and that these levels were associated with resistance of T cells to apoptosis (Sharief and Semra, 2001a; Sharief and Semra, 2001b). Moreover, a very recent study has shown that BIRC2 and BIRC3 mRNA levels were elevated in T cells in active MS patients (Hebb et al., 2008). In the present study, we could not find any differences in the gene expression levels of BIRCs between MS patients and controls, but a negative correlation between the expression of BIRC2 and BIRC3

with volumes of T1 and FLAIR lesions is consistent with the presence of inflammatory activity in the brain. It is noteworthy that the expression of FAS genes also correlated with the number of relapses and the volumes of FLAIR lesions. FAS-FASL signaling plays a critical role in controlling the immune system, indicating the importance of FAS in inflammation (Zipp, 2000).

Taken together, this thesis showed that MS disease activity was associated with upregulation of serum MIF and TRAIL and suggests that these molecules may be candidate biomarkers for disease activity. These findings need to be confirmed with larger patient cohorts and longer follow-up times.

1.2.2 Candidate biomarkers of disease progression

The levels of sFas increased during the follow up in those patients whose EDSS score and T1 lesion volumes increased. T1 lesions represent axonal loss that contributes to the neurological disability in MS (Tomassini and Palace, 2009). Evidence on the predictive value of Fas in the evaluation of disease course has been contradictory (Zipp et al., 1998b; Lopatinskaya et al., 2006). One study reported an association between the increased levels of sFas in sera of patients with short-term disease progression (Zipp et al., 1998b), while a recent 10-year follow-up study reported an association between increased blood mRNA expression with favorable disease course in RRMS and SPMS (Lopatinskaya et al., 2006). Our observations of increased sFas in worsening patients support the concept of sFas promoting autoreactive T-cell survival that in turn is likely to facilitate neurodegenerative events. Moreover, the baseline EDSS score correlated with the expression of TRAIL mRNA and serum MIF and clinical disability progression at one year were associated with the baseline levels of MIF.

It is important to recognize those patients who have a high risk of disability progression. Therefore biomarkers that would predict emergence of disability in invidual MS patient would allow more focused treatment decisions for those patients. These studies showed that disability progression was associated with increased TRAIL mRNA, MIF and sTRAIL and suggest that these molecules may be candidate biomarkers for disability progression.

1.2.3 Conversion-related markers

Prognostic markers predicting the risk of transition from CIS to CDMS would have a strong influence on treatment decisions. Up to now, lesion load in the initial MRI of CIS patients and OCB in CSF has been the best validated prognostic paraclinical measure (Achiron and Barak, 2000; Brex et al., 2002; Fisniku et al., 2008; Rojas et al., 2010).We evaluated whether the changes in apoptosis-related genes are already detectable after the first demyelinating event, in which case they might be used as prognostic markers of a transition to definite MS. Hierarchical cluster analysis showed upregulation of several genes in CIS patients, and three of these genes were even more upregulated in those subjects who converted to definite MS. These three genes can activate (*APAF*) or inhibit (*BIRC6, CFLAR*) caspases, thus promoting proinflammatory events that may be responsible for immune cells' transmigration to the CNS. All these upregulated genes could be useful in the prediction of conversion in the early phase of the disease. Since only a limited number of patients could be included in this exploratory study, the data obtained should be interpreted with caution. These preliminary interesting findings need to be confirmed by larger patient series with longer follow-up periods.

2. Reconstitution of immune activity after pregnancy (Study IV)

Pregnancy influences disease disease activity of MS patients. The relapse rate declines during late pregnancy and increases again after delivery (Confavreux et al., 1998). In spite of recent progress in the understanding of the modulatory effect of pregnancy on MS, very little is known about the detailed molecular mechanisms of this phenomenon (Offner and Polanczyk, 2006). In the present study we analyzed whether the activation of MS after delivery is associated with quantitative changes of apoptotic molecules in serum. The levels of serum sTRAIL and sFasL increased

postpartum compared to late pregnancy samples both in MS and controls. In line with this observation we earlier reported an association between elevated sTRAIL and disease activity in RRMS (Rinta et al., 2008). TRAIL inhibits proliferation of activated T cells (Lunemann et al., 2002; Diehl et al., 2004) and it has been shown to have a beneficial effect in the EAE model (Hilliard et al., 2001). However, in the CNS, TRAIL mediates apoptosis of brain cells (Nitsch et al., 2000; Dorr et al., 2002; Aktas et al., 2005; Dorr et al., 2005). Postpartum upregulation of sTRAIL in both MS patients and controls may be associated with the physiological immune activation known to occur after pregnancy and may reflect a tendency of the immune system to control inflammatory responses**.** The smaller increase of sTRAIL from late pregnancy to postpartum in MS compared to controls may be a factor associated with inadequate inhibition of T-cell reactivation after pregnancy.

The significant increase in the levels of sFasL from pre- to postpartum periods in both patients with MS and their controls may be associated with increased immune activation. This is in accordance with our data showing increased levels of sFasL during MS relapses (Rinta et al., 2008) and results by other investigators reporting increased expression of FasL mRNA in blood cells during lesional activity (Lopatinskaya et al., 2003). The similar levels of sFas in pre- and postpartum samples of MS patients are consistent with previous data by Ehrlich et al (Ehrlich et al., 2007).

Currently, we cannot predict which patients disease activity will increase after delivery. Therefore it would be important to find a biomarker that is associated with the postpartum disease activation. That would help mothers to decide whether initiate early DMT to minimize risk of relapses or continue breast-feeding. In this study, we showed that there was a slight difference in the levels of ΔsFasL in those patients who relapsed after delivery and those who remained stable.

Taken together, upregulation of sTRAIL and sFasL from pregnancy to the postpartum situation both in patients with MS and healthy women is the main observation of our study. Since these molecules are secreted by immune cells in activation-dependent manner, it is most likely that the quantitative changes detected in this study are due to T-cell activation occurring during physiological reactivation of the mother's immune system after pregnancy (Elenkov et al., 2001; Gilmore et al., 2004). To clarify whether the TRAIL and sFasL could be used as a postpartum disease activity marker, this study needs to be repeated with the larger patient cohort.

SUMMARY AND CONCLUSIONS

The aim of this study was to identify biomarkers in the blood that would reflect pathogenetic processes in MS and CIS and be used as biomarkers of disease activity and progression.

The major findings of the study were:

1. Altered expression of immune profiles in MS subtypes was found in both protein and gene expression levels. In PPMS, elevated sFas, TNF-α and CCL2 concentrations is consistent with the presence of inflammatory activity in this subtype. Therefore, identification of immunological subtypes more carefully in this disease form may allow development of immunomodulatory therapies for PPMS patients. In RRMS and CIS, atypical gene expression profiles involving genes of Bcl-2 and NF-κB families and death receptor pathway were identified. Upregulation of these pro- and anti-apoptosis molecules indicate the regulatory effect of the immune system in maintaining peripheral homeostasis and thereby preventing pathogenetic events.

2. MS disease activity was associated with upregulation of serum MIF and, TRAIL, and disease progression was associated with increased TRAIL mRNA, MIF and sFas. These observations suggest that these molecules may be candidate biomarkers for disease activity and progression.

3. In CIS, three out of 93 apoptosis-related genes were upregulated in those subjects who converted to definite MS. These three genes can activate (*APAF*) or inhibit (*CFLAR, BIRC6*) caspases. These upregulated genes could be useful in the prediction of conversion to MS.

4. sTRAIL and sFasL were upregulated in both MS patients and healthy women from late pregnancy to postpartum. The increase in sTRAIL was significantly smaller in the MS patients compared with the controls. Hence, activation of MS after delivery may be related to inadequate inhibition of T-cell reactivation after pregnancy in MS.

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Apoptosis-related molecules in blood in multiple sclerosis

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TNF-related apoptosis-inducing ligand

ABSTRACT

A failure in apoptosis of lymphocytes may lead to harmful immunoreactivity in MS. We analyzed apoptosis-related molecules including TRAIL, sFas, sFasL and MIF in the blood of 117 MS patients and controls to answer whether these molecules may be used in the evaluation of disease activity and immunomodulatory effect of IFN-β. Increased levels of sTRAIL, sFasL and MIF were found in sera of untreated patients with MS relapse indicating their association with MS disease activity. IFN-β treated patients in remission had increased TRAIL mRNA, sTRAIL, sFaL and MIF that most likely reflect bioactivity of IFN-β.

1. Introduction

Multiple sclerosis (MS) is an immune-mediated disease of the central nervous system (CNS) initiated by recruitment of activated T cells and macrophages to the brain (McFarland and Martin, 2007). A failure of autoreactive immune cells to undergo apoptosis in MS may lead to inappropriate persistence of these cells and cause harmful immunoreactivity within the CNS (Okuda et al., 2006; Saresella et al., 2005; Sharief, 2000; Wosik et al., 2007). Since the presence of apoptosis-related molecules in blood is thought to reflect the pathological process in the CNS, determination of these molecules may be useful in evaluation of disease activity and therapeutic responses.

Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a new molecule among the potential biomarkers in MS (Gilli et al., 2006; Wandinger et al., 2003). Upregulated expression of TRAIL messenger RNA (mRNA) has been reported in peripheral blood mononuclear cells (PBMC) of interferon-β (IFN-β) treated relapsing remitting MS (RRMS) patients responding to treatment (Gilli et al., 2006; Wandinger et al., 2003). Based on these observations it has been suggested that TRAIL could be used as a biomarker reflective of response to IFN-β treatment in MS (Wandinger et al., 2003). In experimental disease models including experimental autoimmune encephalomyelitis (EAE), HIV encephalopathy and stroke, TRAIL has been shown to mediate apoptosis of brain cells (Aktas et al., 2005; Martin-Villalba et al., 1999; Miura et al., 2003).

Fas ligand (FasL/CD154) is another member of the TNF family that together with its receptor Fas (CD95), participates in the apoptotic signalling pathway in the activated T cells (Askenasy et al., 2005). Increased mRNA expression of Fas and FasL has been consistently reported in the PBMC of RRMS patients (Gomes et al., 2003; Huang et al., 2000), but the results on the levels of soluble Fas (sFas) in sera are contradictory (Bilinska et al., 2003; Inoue et al., 1997; Mahovic et al., 2004; Zipp et al., 1998b). Membrane-bound forms of Fas and FasL are able to induce apoptosis of T cells, while soluble forms inhibit apoptotic events. This suggests that upregulated levels of sFas and sFasL may prevent apoptosis of potentially pathogenic immune cells, which may lead to their accumulation in the CNS.

Macrophage migration inhibitory factor (MIF), involved in many inflammatory diseases, is secreted by activated T cells, macrophages and a variety of nonimmune cells (Cvetkovic and Stosic-Grujicic, 2006). MIF is a pleiotropic inflammatory cytokine with broad target cell specificity. MIF upregulates expression of adhesion molecules, proinflammatory cytokines and toxic molecules (Cvetkovic and Stosic-Grujicic, 2006). Increased expression of MIF has been reported in the cerebrospinal fluid (CSF) of patients with MS relapses (Niino et al., 2000). Anti-MIF treatment of mice with EAE decreased accumulation

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of encephalitogenic T cells in the central nervous system (CNS), ameliorated disease severity and accelerated recovery from the disease (Denkinger et al., 2003).

The aim of this study was to examine whether the expressions of TRAIL gene and the levels of sTRAIL, sFas, sFasL and MIF in sera of MS patients may be used in the evaluation of disease activity and response to IFN-β treatment in MS.

2. Subjects and methods

2.1. Subjects

The study included 117 subjects: 70 RRMS patients, 21 secondary progressive MS (SPMS) patients and 26 healthy controls. Thirty seven of 91 MS patients were treated with IFN-β and the remaining ones were untreated. All IFN-β products were used: Avonex® (30 μg IFN-β-1a intramuscular once a week), Rebif® (22 μg or 44 μg IFN-β-1a subcutaneously (sc) three times a week) and Betaferon® (250 μg IFN-β-1b sc every other day). Serum samples from 10/37 IFN-β treated patients and 22/54 untreated patients were obtained at the time of acute relapse before initiation of methylprednisolone (MP) treatment. The clinical characteristics of these patients are shown in Table 1. The diagnosis of MS was based on McDonald's criteria and all diagnoses were definite (McDonald et al., 2001). Neurological disability was evaluated by the Expanded Disability Status Scale (EDSS) (Kurtzke, 1983). None of the patients had received MP during the eight weeks prior to study entry. The study was approved by Ethics Committee of Tampere University Hospital and all subjects gave written informed consent.

Based on clinical characteristics, IFN-β treated patients in remission were divided into responders ($n=16$) and partial responders ($n=7$). Those subjects who had no further relapses and were without deterioration in the EDSS during one-year IFN-β treatment were defined as drug responders and the remaining ones as partial responders. Four patients that had been on IFN-β treatment for less than three months could not be included into this subanalysis. Since the evaluation of clinical efficiency of IFN-β treatment was not the primary aim of this study, the presence of neutralizing antibodies was not obtained and the timing of IFN-β injection prior to blood sampling was not recorded.

Serum measurements were performed in 91 MS patients and 26 healthy controls (Table 1). Expression of TRAIL mRNA was analyzed from the PBMC of 46 MS patients and 17 healthy controls (Table 2), while membrane expression of TRAIL was analyzed in 9 RRMS patients and 6 healthy controls.

Table 2
Clinical characteristics of patients included in TRAIL mRNA analyses

Characteristic	All MS patients	MS		Healthy controls
		No treatment	IFN-β	
	$N=46$	$N=23$	$N=23$	$N=17$
Disease course (RR/SP)	36/10	15/8	21/2	NA
Age (years)[a]	39.7±9.7	41.8±18.2	35.4±9.0	35.4±9.4
Gender (F/M)	32/14	15/8	17/6	17/9
Duration of disease (years)[a]	7.3±6.3	4.3±3.6	6.9±6.6	NA
No. of relapses/2 years[a]	1.6±1.5	1.1±1.1	1.7±1.1	NA
EDSS[a]	3.0±2.0	2.3±1.9	1.9±2.0	NA
Progression index[a,b]	1.3±2.2	1.1±1.6	0.3±0.4	NA

RR relapsing remitting multiple sclerosis, SP secondary progressive MS, IFN-β interferon-β, NA not available, EDSS expanded disability status scale.
[a] Mean±SD.
[b] EDSS/duration of the disease.

2.2. Total RNA isolation from PBMC and serum separation

PBMC were separated in a VACUTAINER CPT cell preparation tube (Becton Dickinson and Company, Franklin Lakes, N.J., USA) according to the manufacturer's protocol. Total cellular RNA was isolated from stored cell lysate with Qiagen's RNase minikit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. DNA was removed with Qiagen's RNase-Free DNase set. Total RNA was eluted to nuclease-free water and samples were stored −70 °C. Sera were separated from blood and stored at −70 °C.

2.3. Relative quantitative RT-PCR

The expression of TRAIL mRNA could be determined in 46 patients and 17 controls in which RNA was available (Table 2). A relative quantitative real time polymerase chain reaction (RT-PCR) was performed with a LightCycler instrument (Roche Diagnostics GmbH, Mannheim, Germany). 1 μg of total RNA was reverse transcribed to complementary DNA (cDNA) with a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) following standard protocol with random hexamer primers. 2-μl cDNA was used in a total of 20 μl reaction volume for the PCR reactions. The final concentrations of the target reagents in the system were: 1×LightCycler FastStart DNA Master HybProbe, 4 mM MgCl2, 0,5 μM each primer and 0,2 μM each probe. PCR cycling conditions were: denaturation at 95 °C for 10 min, amplification 45 cycles, at 95 °C for 15 s, at 55 °C for 15 s and at 7 °C for 15 s and cooling at 40 °C for

Table 1
Clinical characteristics of MS patients ($n=91$) and healthy controls ($n=26$)

Characteristic	All MS patients[a]	Remission		Relapse		Healthy controls
		No treatment	IFN-β[b]	No treatment	IFN-β[c]	
	$N=91$	$N=32$	$N=27$	$N=22$	$N=10$	$N=26$
Disease course (RR/SP)	70/21	20/12	25/2	17/5	8/2	NA
Age (years)[d]	40.0±12.2	42.5±16.1	35.6±9.4	40.3±9.0	37.3±8.5	36.0±9.7
Gender (F/M)	63/28	21/11	20/7	16/6	4/6	17/9
Duration of disease (years)[d]	6.4±5.9	5.4±5.7	7.0±6.3	6.7±6.3	7.9±4.1	NA
No of relapses/2 years[d]	1.7±1.6	0.9±1.1	1.6±1.2	2.0±1.7	3.4±2.2	NA
EDSS[d]	2.4+2.0	2.6±2.1	1.9±1.8	2.6±1.3	3.2±1.8	NA
Progression index[d,e]	0.89±1.6	1.1±1.5	0.34±0.4	4.0±4.4	0.5±0.4	NA
Duration of treatment (years)[d]	2.6±2.4	NA	2.3±2.4	NA	3.4±2.2	NA

RR relapsing remitting MS, SP secondary progressive MS, IFN-β interferon-β, NA not available, EDSS expanded disability status scale.
[a] 37 of the 91 patients were treated with IFN-β and the remaining ones were without immunomodulatory treatment.
[b] Patients had the following treatments: Avonex® ($n=7$), Betaferon® ($n=11$) and Rebif® ($n=9$).
[c] Patients had the following treatments: Avonex® ($n=2$), Betaferon® ($n=3$) and Rebif® ($n=5$).
[d] Mean±SD.
[e] EDSS/duration of the disease.

30 s. Primers and probes for the LightCycler PCR were designed and prepared by TIB MolBiol (Berlin, Germany). The following TRAIL primers and probes were used: forward primer: 5′-AGAGAGTATGAA-CAGCCCCTG-3′, reverse primer: 5′-GTCCCAGTTATGTGAGCTGCTA-3′, Probe-5′-end labelled with acceptor dye Red 640: 5′-TTCTAACGAGCT-GACGGAGTTGCCA-3′ and probe 3′-end labelled with fluorescein: 5′-GGTTTCCTCAAGAGGTTCTCAAAAATCATC-FL-3′. As a reference gene we used human glucose-6-phosphate-dehydrogenase (h-G6PDH). Reference gene PCR was done at the same time and the same PCR conditions as the target gene by a LightCycler® h-G6PDH housekeeping gene set kit (Roche Diagnostic GmbH, Mannheim, Germany). The reference gene was selected by a LightCycler h-Housekeeping Gene Selection Set, which included five alternative reference genes. All reactions for target and reference genes were made as duplicate. Quantitative RT-PCR results were calculated by a LightCycler Relative Quantification Software with efficiency correction (Roche Diagnostics GmbH).

2.4. Determination of soluble TRAIL in sera

Levels of sTRAIL in undiluted sera were quantified by a Diaclone solid phase sandwich ELISA kit (# 850.770.096; Diaclone, Besancon cedex, France). Detection limits were 64 pg/ml. TRAIL absorbancies were read with a Multiskan MS version 4.0 spectrophotometer (Labsystems, Helsinki, Finland) at wavelength 450 nm.

2.5. Determination of soluble Fas, FasL and MIF using Bio-Plex suspension array system

The levels of sFas, sFasL and MIF were measured simultaneously by Bio-Plex suspension array system (Bio-Rad laboratories, Hercules, CA, USA). A multiplex cytokine analysis kit was obtained from Linco Research, Inc. (St. Charles, MO, USA). Samples were diluted 1 to 10 in serum matrix and were run in duplicate according to the manufacturer's protocol. Data were collected and analyzed using the Bio-Plex ManagerTM software 4.1 (Bio-Rad laboratories). A five-parameter regression formula was used to calculate the sample concentrations from the standard curve. The minimum detection limit for all the cytokines was 12.2 pg/ml.

2.6. Cell-surface expression of TRAIL by multicolor FACS analysis

PBMC for TRAIL membrane expression analyses was obtained from 9 RRMS patients and 6 healthy controls. PBMCs (2×10^5) were incubated with mouse anti-human CD3-APC-Cy7, CD4-PE-Cy7, CD8-APC, CD14-FITC and TRAIL-PE (Becton Dickinson, Franklin Lakes, NJ, USA) monoclonal antibodies for 20 min. As an isotype control, cells were incubated with mouse anti-human IgG1-APC-Cy7, IgG1-PE-Cy7, IgG1-APC, IgG1-FITC and IgG1-PE monoclonal antibodies. Thereafter cells were washed twice, resuspended in PBS, and analyzed with FACSAria flow cytometry and Facsdiva software (Becton Dickinson). Stainings were made in duplicate. Optimization of fluorescence compensation was made using BD CompBeads (Becton Dickinson).

2.7. Statistical analyses

Statistical analyses were performed using SPSS version 14.0 for Windows (SPSS Inc., Chicago, Illinois, USA). Results of TRAIL mRNA were normally distributed and group comparisons were made using the independent samples *T*-test. Due to the skewed distributions of serum cytokine levels, the group comparisons were made using the Kruskal–Wallis and Mann–Whitney *U* test. A *p* value less than 0.05 was considered statistically significant. Correlations between variables were tested by two-tailed non-parametric Spearman correlation analysis. Due to multiple correlation testing, a *p* value of less than 0.01 was considered significant.

3. Results

3.1. Expression of apoptosis-related cytokines in MS and controls

The expression analysis of TRAIL mRNA performed on 46 MS patients and 17 healthy controls showed that the levels of this cytokine were of the similar magnitude (Table 2, Fig. 1). Measurement of soluble molecules performed from sera of 91 MS patients and 26 controls (Table 1 and Fig. 2) revealed higher levels of sTRAIL in MS compared to controls (median: 1082.3 vs. 719.0 pg/ml, $p=0.01$), but no differences were found in the levels of sFas, sFasL or MIF.

3.2. Relationship to MS disease activity

These analyses included 22 untreated MS patients with relapse, 32 untreated patients in remission and 26 controls (Table 1). Comparison between patients at relapse with controls showed increased sTRAIL (1374.3 vs. 719.0 pg/ml: $p<0.001$, and MIF (888.9 vs. 299.4 pg/ml, $p<0.001$) in patients with relapse (Fig. 2A and D). The levels of these cytokines in patients in remission and controls were without significant differences. Likewise, the levels of sFas and sFasL were without differences between all studied groups.

Comparison between untreated patients with relapse and remission revealed higher levels of sTRAIL (1374.3 vs. 726.6 pg/ml, $p<0.001$) and MIF (888.9 vs. 150.4 pg/ml, $p<0.001$) in patients with relapse (Fig. 2A and D). However, the levels of sFas and sFasL were without significant differences between these two patient groups (Fig. 2B and C). Comparison between RRMS patients at relapse ($n=17$) and remission ($n=20$) showed increased levels of sFasL in patients with relapse (98.0 vs. 52.0, pg/ml, $p=0.002$). No differences were found in the expression of TRAIL mRNA between patients in remission and controls (Fig. 1).

3.3. Relationship to IFN-β treatment

These analyses included 37 IFN-β treated patients (27 in remission and 10 with relapse) and 54 untreated patients (32 in remission and 22 with relapse) (Table 1). Higher expression of TRAIL mRNA was found in IFN-β treated patients compared to untreated ones (2.0±1.3 vs. 1.3±0.4; $p=0.03$, Fig. 1). Moreover, comparison between IFN-β treated and untreated patients with remission showed higher levels of sTRAIL (999.0 vs. 726.6 pg/ml, $p=0.004$), sFasL (median: 100.0 vs. 65.5 pg/ml, $p=0.04$) and MIF (median: 414.9 vs. 150.4 pg/ml, $p=0.02$) in those that were treated with IFN-β. But no differences were found in the levels of sFas (Fig. 2). Comparison between treated and untreated patients at relapse did not reveal differences in the levels of any apoptotic cytokine.

Comparison between IFN-β responders ($n=16$) and partial responders ($n=7$) showed elevated levels of sFas (5633.4 vs. 4148.7 pg/ml,

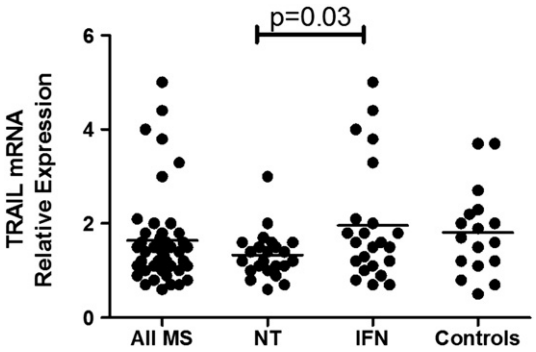

Fig. 1. TRAIL mRNA expression in 46 all MS patients, 23 non-treated (NT), 23 IFN-β treated MS patients (IFN) and 17 healthy controls (mean±SD). Relative expression of TRAIL mRNA is shown for each donor.

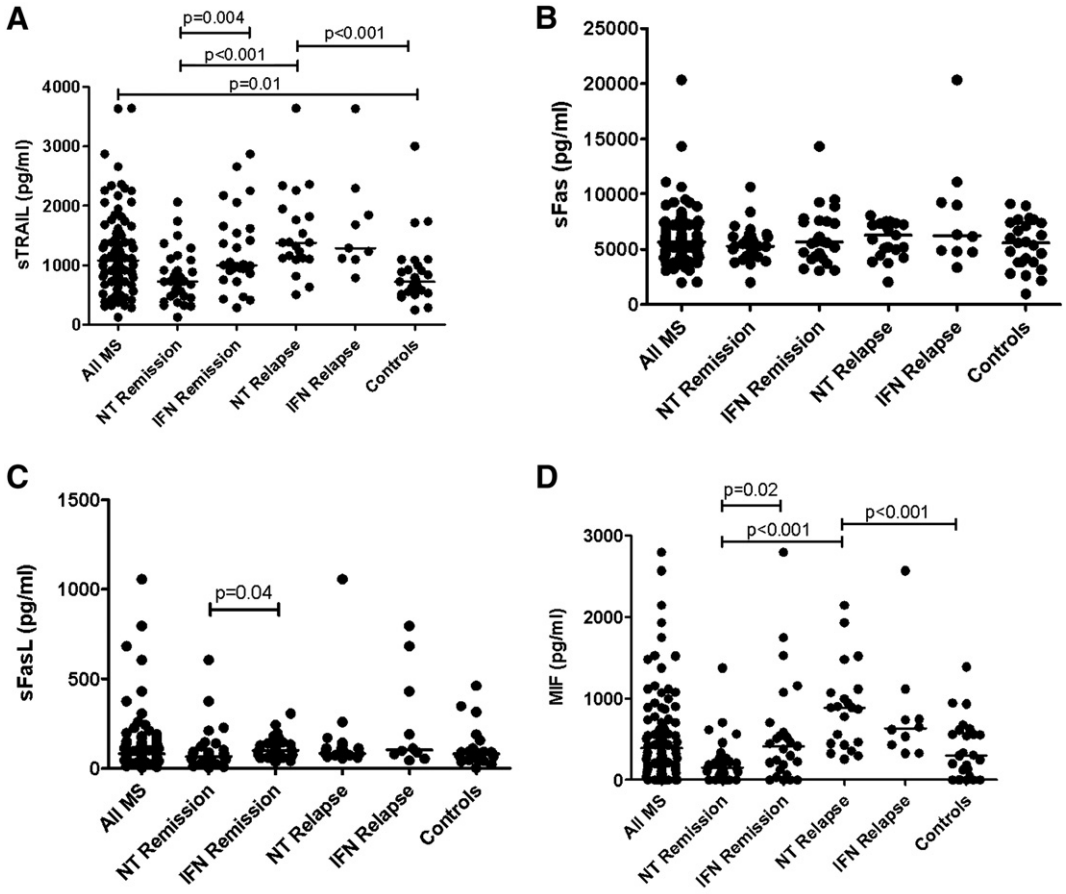

Fig. 2. The median levels of (A) sTRAIL, (B) sFas, (C) sFasL and (D) MIF in sera of all MS patients (n=91), untreated MS patients during remission (n=32), IFN-β treated MS patients during remission (n=27), untreated MS patients during relapse (n=22), IFN-β treated MS patients during relapse (n=10) and healthy controls (n=26). The levels of cytokines are shown for each donor. IFN = interferon-β, NT = no treatment.

p=0.04) in IFN-β responders (Fig. 3). No differences in the levels of TRAIL mRNA (2.2±1.4 vs. 1.8±1.0), sTRAIL (1291.7 vs. 981.5 pg/ml), sFasL (100.0 vs. 78.4 pg/ml) and MIF (507.4 vs. 227.4 pg/ml) were found between these patients groups.

3.4. TRAIL membrane expression

No differences in the surface expression of TRAIL on CD4+ and CD8+ T cells and CD14+ monocytes was found in the 9 MS patients and 6

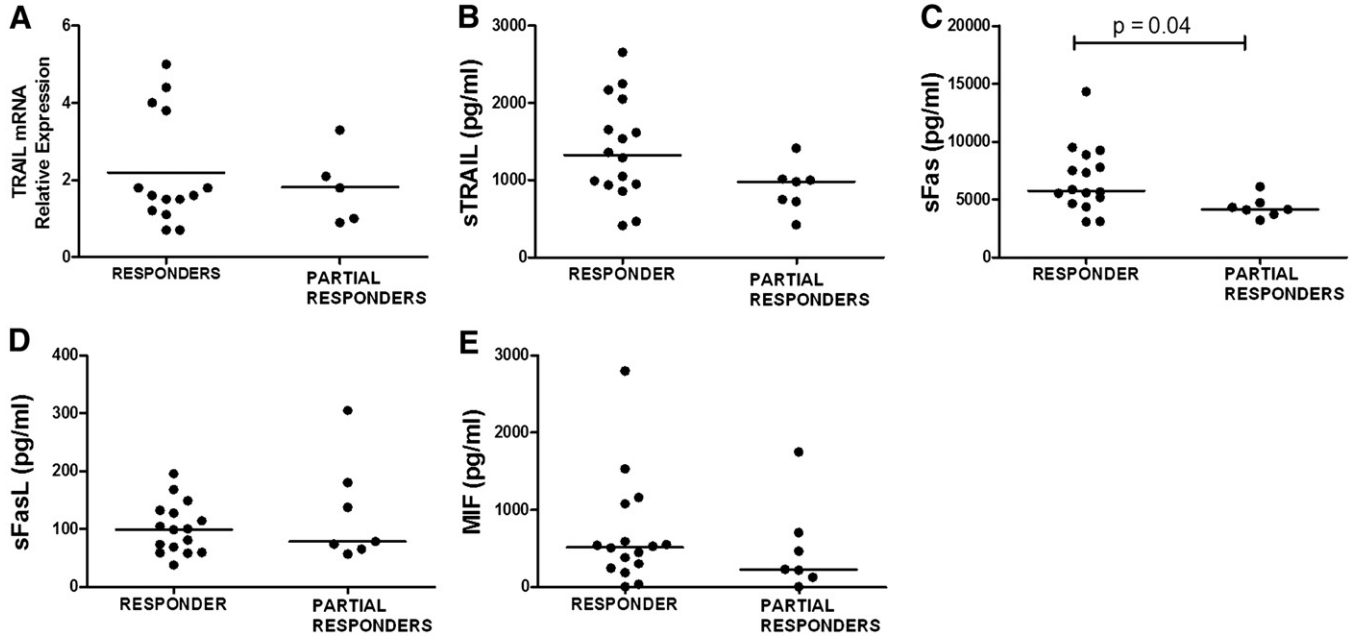

Fig. 3. The levels of (A) TRAIL mRNA, (B) sTRAIL, (C) sFas, (D) sFasL and (E) MIF in INF-β responders and partial responders. The levels of apoptotic molecules are shown for each donor.

healthy controls that could be included in these analyses. The surface expression of TRAIL was detectable in less than 1% of CD3+CD4+ or CD3+CD8+ T cells and in less than 5% of monocytes (Fig. 4).

3.5. Correlations

The clinical relevance of detected immunological findings was explored by correlating these data to clinical characteristics of the patients. In the group including all 91 MS patients the expression of TRAIL mRNA correlated positively with EDSS (r=0.38, spearman p=0.01). In the IFN-β treated RRMS patients in remission (n=21) the expression of TRAIL mRNA correlated positively with both EDSS (r=0.59, spearman p=0.005) and progression index (r=0.56, spearman p=0.009, Fig. 5A and C).

4. Discussion

In MS a failure in apoptosis of autoreactive T cells may lead to increased immunoreactivity within the CNS that may damage myelin, oligodendrocytes, and neurons (Zipp, 2000). Since the presence of apoptosis-related molecules in peripheral blood may reflect apoptotic events in the CNS, determination of such molecules may be useful in evaluation of disease activity and response to treatment. The aim of this study was to examine whether the expressions of the TRAIL gene and the levels of sTRAIL, sFas, sFasL and MIF in sera of MS patients may be used for these purposes and whether the IFN-β treatment modulates their expressions. In this study we found increased levels of sTRAIL, sFasL and MIF in untreated patients with MS relapse, indicating an association of these molecules with MS disease activity. The upregulated levels of TRAIL mRNA, sTRAIL, sFasL and MIF IFN-β treated patients most likely reflecting the bioactivity of this treatment.

Until now the results on TRAIL mRNA and sTRAIL in sera of untreated MS patients have been contradictory (Buttmann et al., 2007; Gilli et al., 2006; Huang et al., 2000). Upregulated expression of TRAIL mRNA in RRMS has been reported by Huang et al., but this could not be confirmed by others. The lack of differences in the levels of TRAIL mRNA or sTRAIL in MS patients and controls detected in this study is in line with previous reports. In addition, the upregulated levels of sTRAIL seen in patients at relapse indicate that determination of this cytokine may be useful in the evaluation of MS disease activity. TRAIL is an anti-inflammatory molecule, upregulated TRAIL in sera may exert neuroprotective effects through inhibition of proinflammatory activity during relapse. However, this concept is in contrast with an observation of apoptosis-inducing effects of TRAIL in human brain reported by Nitsch et al. (2000). The expression of TRAIL on the surface of T cells and monocytes from blood of MS patients and controls was less than 5%, corresponding to earlier data showing low expression of TRAIL in freshly isolated PBMC (Arbour et al., 2005; Kayagaki et al., 1999).

MIF has been reported to upregulate expression of adhesion molecules, proinflammatory cytokines and toxic molecules thus facilitating penetration of autoreactive T cells into the CNS with following induction of apoptosis of neural tissue (Cvetkovic and Stosic-Grujicic, 2006). Increased level of MIF in sera of our patients at the time of MS relapse is consistent with proinflammatory activity of this cytokine. This observation is also in line with data by Niino et al. who showed upregulated levels of MIF in the CSF of MS patients with acute relapse (2000).

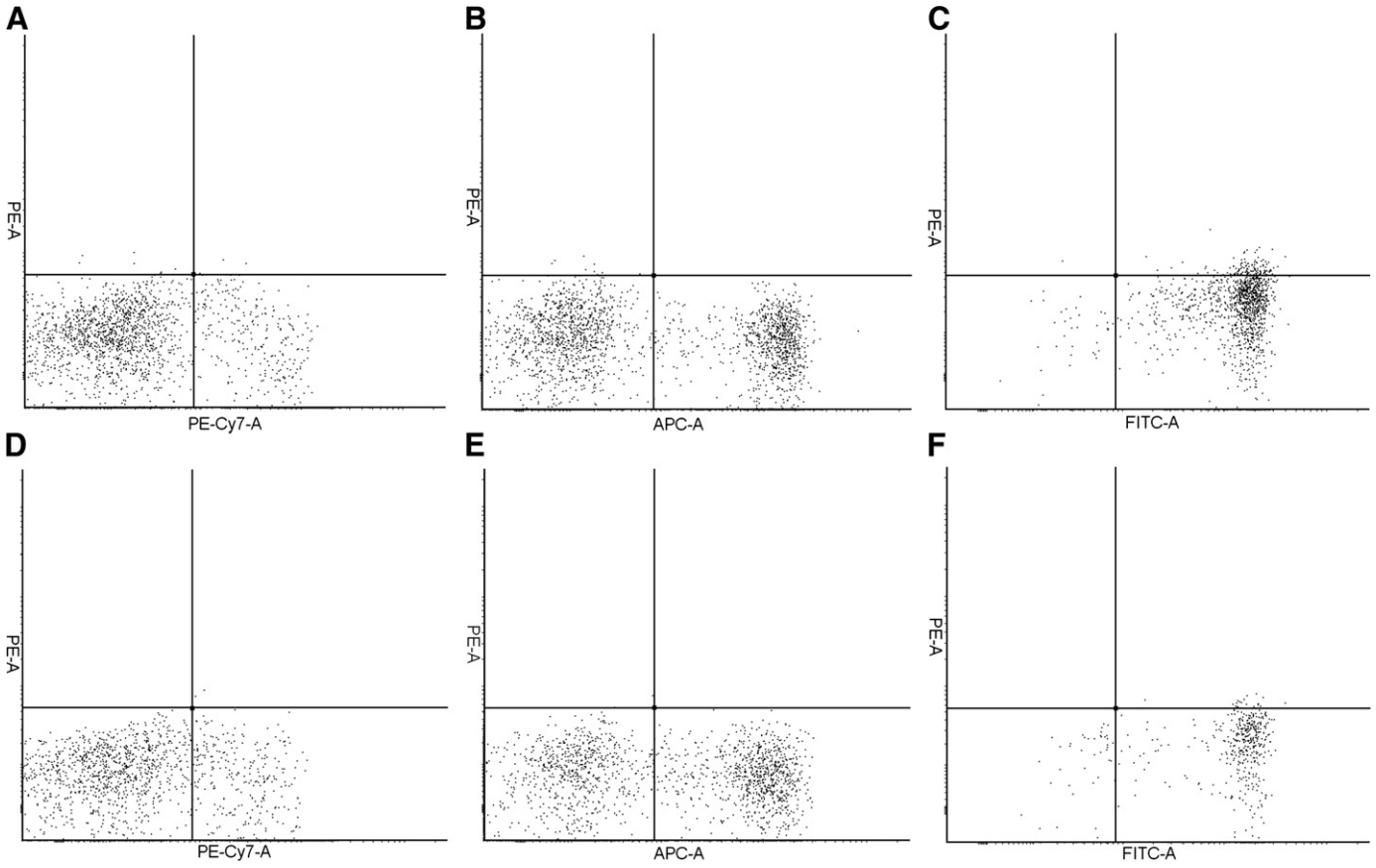

Fig. 4. Expression of membrane-bound TRAIL in freshly isolated PBMC from MS patients and controls. Lymphocyte and monocytes populations were identified in the total PBMC population according to forward light scatter and side scatter parameters. CD3+ population was gated from histograms and (A) CD3+CD4+TRAIL+ cell populations, (B) CD3+CD8+TRAIL+, (C) CD14+TRAIL+, (D) CD3+CD4+TRAIL+, (E) CD3+CD8+TRAIL+, and (F) CD14+TRAIL+ populations shown in the figure from RRMS patient (A–C) and healthy control (D–F). These profiles are representative analyses.

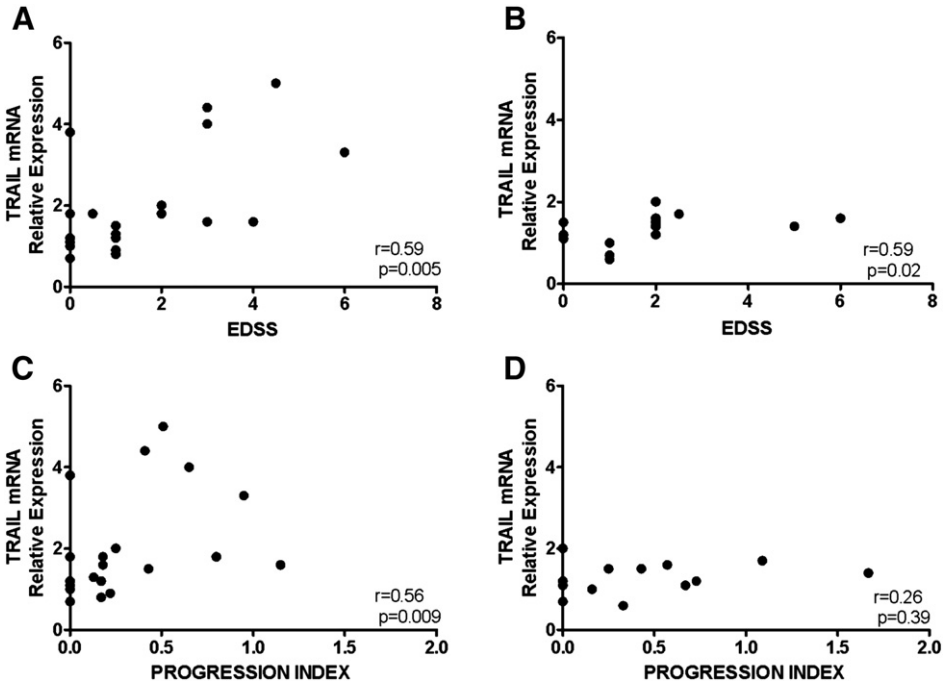

Fig. 5. Correlations between EDSS score and TRAIL mRNA in (A) IFN-β treated RRMS and (B) untreated RRMS patients and progression index and TRAIL mRNA in (C) IFN-β treated RRMS and (D) untreated RRMS patients.

Fas–FasL is one of the death receptor–ligand systems of T cells that is crucial in the apoptotic signalling pathway (Askenasy et al., 2005). Defective function of Fas in T cells in MS has been reported earlier (Comi et al., 2000; Okuda et al., 2006), but the exact role of sFas and sFasL in pathogenesis of MS is still not known. Increased level of sFasL in sera of RRMS patients with relapse detected in this study suggests a failure in Fas–FasL apoptosis pathway of autoreactive T cells. This in turn may facilitate accumulation of these T cells into the CNS with subsequent development of clinical disease activity. It is noteworthy that increased expression of FasL mRNA in blood cells during lesional activity has been earlier reported (Lopatinskaya et al., 2003). The lack of differences in the levels of sFas between patients with MS relapse and remission and between MS patients and controls found in the present study is consistent with results by Ciusani et al. (1998) and Inoue et al. (1997), but in contrast with those of Zipp et al. (1998b) who reported elevated levels of sFas in sera in RRMS. The control group in the study by Zipp et al. included patients with other neurological diseases in addition to healthy individuals, which may be a factor explaining the discrepancy.

In the present study we were able to confirm previous observations of upregulated sTRAIL in IFN-β treated patients (Buttmann et al., 2007; Gilli et al., 2006; Santos et al., 2006; Wandinger et al., 2003). In addition, elevated levels of sFasL and MIF were detected in IFN-β treated patients. The presence of upregulated apoptotic molecules in sera of IFN-β treated patients most likely reflects immunomodulatory activity of IFN-β. Based on these results inhibition of activated T cells by TRAIL could be one of the mechanisms responsible for beneficial effect of IFN-β. Increased levels of sFasL and MIF in IFN-β treated patients in remission compared to untreated MS group is most likely due to more active disease in treated group (1.6 relapses in IFN-β group vs. 0.9 relapses in untreated group before 2 years preceding sampling). The presence of similar levels of sFas in IFN-β treated and untreated MS patients is in line with earlier reports thus suggesting that initiation of IFN-β therapy causes only transient increase in levels of circulating sFas (Boylan et al., 2001; Zipp et al., 1998a).

The results on the significance of TRAIL in evaluation of treatment responses have been contradictory (Buttmann et al., 2007; Gilli et al., 2006; Santos et al., 2006; Wandinger et al., 2003). Wandinger et al. reported that TRAIL could be used as a response marker for IFN-β therapy (Gilli et al., 2006; Wandinger et al., 2003), but others have not been able to confirm this observation (Buttmann et al., 2007; Gilli et al., 2006). In the present cross-sectional study we could not differentiate between responders and partial responders to IFN-β based on the levels of TRAIL mRNA and sTRAIL in these groups. This is accordance with Buttmann et al. (2007). Correlation between TRAIL mRNA and EDSS or progression index in IFN-β treated RRMS patients in remission found in this study contradicts with current understanding of neuroprotective effects of TRAIL, but this observation needs to be confirmed by other studies.

In conclusion, the present study was undertaken to explore whether the expressions of the TRAIL gene and the levels of sTRAIL, sFas, sFasL and MIF in the blood of MS patients may be used in evaluation of disease activity and immunomodulatory effect of IFN-β treatment. Based on our data upregulated sTRAIL, MIF and sFasL in MS patients with relapse reflect increased clinical disease activity. In IFN-β treated patients increased levels of TRAIL, MIF and FasL are indicators of bioactivity of this drug.

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Disease-associated inflammatory biomarker profiles in blood in different subtypes of multiple sclerosis: Prospective clinical and MRI follow-up study

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ABSTRACT

To identify biomarkers of disease activity and progression in multiple sclerosis (MS), we analyzed the serum profiles of cytokines, chemokines and apoptotic molecules in different subtypes of MS including clinically isolated syndrome (CIS) and correlated their levels with clinical and volumetric MRI findings obtained over a one-year follow up. Upregulated levels of apoptotic sFas molecule were found in MS patients with a worsening EDSS score and an accumulation of hypointense lesions in MRI. In such patients, the levels of MIF appeared to be higher than in non-progressing patients. In addition, increased levels of serum TNF-α and CCL2 were found especially in primary progressive MS (PPMS). These observations suggest that serum Fas and MIF are candidate biomarkers of neurological worsening related to progressive neurodegeneration, while serum TNF-α and CCL2 reflect the presence of inflammatory responses in PPMS.

1. Introduction

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) that is characterized by complex pathophysiological processes including inflammation, demyelination, axonal loss and remyelination (McQualter and Bernard, 2007). Currently, the clinical assessments used in the treatment of MS are relatively crude and depend mainly on relapses and changes in disability scores. Magnetic resonance imaging (MRI) is the main paraclinical tool used in clinical practice (McFarland, 2009), but clinicoradiological correlations have been generally weak thus far (Barkhof, 2002). Likewise, prognostic markers predicting a risk of transition from clinically isolated syndrome (CIS) to clinically definite MS (CDMS) would have a strong influence on treatment decisions. Up to now, lesion load in the initial MRI of CIS patients has been the best validated prognostic paraclinical measure (Brex et al., 2002, Achiron and Barak, 2000, Fisniku et al., 2008). Due to these reasons, sensitive biomarkers are needed for the evaluation of disease activity and progression, predicting the disease course and optimizing therapeutic responses in MS (Tumani et al., 2009, Harris and Sadiq, 2009).

The majority of blood and CSF markers analyzed thus far in MS reflect both immune-inflammatory and neurodegenerative events, but their correlation with clinical aspects have been relatively weak (Tumani et al., 2009, Harris and Sadiq, 2009). Cytokines and chemokines are major regulators of inflammation that mediate the recruitment of leukocytes into CNS with the subsequent development of tissue damage (McQualter and Bernard, 2007). Several chemokines including CCL2, CCL4, CCL5, CXCL10, CXCL12, and CXCL13 and their receptors including CCR1, CCR2, CCR5, CXCR3, and CXCR4 have been detected in the active MS lesions, as well as in CSF and blood where they were considered to reflect disease activity (Szczucinski and Losy, 2007). Moreover, recently expression of CXCR3+ on circulating CD8+ cells was associated with MRI measurements of inflammatory activity and tissue destruction (Fox et al., 2008). Interestingly, it has been reported that interferon (IFN)-β can modulate the levels of several chemokines (CCL1, CCL2, CCL7, CXCL10 and CXCL11) in blood and CSF, thus limiting the entry of immune cells into the CNS (Cepok et al., 2009, Sellebjerg et al., 2009).

Altered levels of proinflammatory cytokines such as interleukin (IL)-2, IL-12, IL-6, tumor necrosis factor (TNF) -α and IFN-γ, and of regulatory cytokines such as IL-10, IL-4 and transforming growth factor (TGF)-β in the blood and CSF of MS patients have also been reported (Imitola et al., 2005). These were considered to reflect the *in vivo* activity of inflammatory cells (Sospedra and Martin, 2005). Some of these cytokines are associated with disease activity on MRI (Fassbender et al., 1998, Petereit et al., 2003). In general, markers of inflammation show rather poor correlation with clinical disease progression and there are no data on any sufficiently long period follow up (Tumani et al., 2009).

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A failure in the apoptotic cell death of autoreactive T cells is an important pathophysiological mechanism in MS (Zipp, 2000, Pender, 2007). Neuropathological evidence showing enhanced expression of death receptors Fas (CD95), TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1), TRAIL-R2, TNF-R1, TNF-R2 and their ligands Fas Ligand (FasL), TRAIL and TNF-α in MS together with expression of apoptotic molecules in blood and CSF favors this concept (D'Souza et al., 1996, Dowling et al., 1996, Bonetti and Raine, 1997, Cannella et al., 2007). However, only a few studies have analyzed the potential of these molecules to predict clinical outcomes of disease.

Our aim in this study was to identify biomarkers in the blood that could reflect pathogenetic processes in MS and be used in clinical practice when treating patients with MS. Therefore, the profiles of several cytokines, chemokines and apoptotic molecules that are considered to be involved in pathogenesis of MS were evaluated with the aim of recognizing such molecules that could be used as biomarkers of disease activity and progression in MS.

2. Patients and methods

2.1. Subjects

The study was a one-year prospective follow-up study that included altogether 110 subjects of whom 72 had CDMS according to revised McDonald Criteria (McDonald et al., 2001, Polman et al., 2005), 17 had CIS and the remaining 21 were healthy controls (HC). The CDMS group included 33 patients with relapsing remitting MS (RRMS), 18 patients with secondary progressive MS (SPMS) and 21 with primary progressive MS (PPMS). CIS patients were defined as patients who had their first demyelinating neurologic event suggestive of MS. Six patients had discontinued the study after the baseline visit because of severe disability (1 RRMS, 1 SPMS and 4 PPMS) and two RRMS patients were not willing to continue. The study was approved by the Ethics Committee of Tampere University Hospital and all subjects gave informed consent. All patients underwent neurological and MRI examination at the baseline and one year after enrollment. The blood was drawn on the same day as the neurological examination. The clinical evaluation included the determination of an Expanded Disability Status Scale (EDSS) score (Kurtzke, 1983), number of relapses 2 years before study entry, disease duration, progression index, and different immunomodulatory treatments that are summarized in Table 1. Age and gender-matched healthy controls were recruited from the staff of the University of Tampere or Tampere University hospital.

2.2. Determination of cytokines, apoptotic molecules and chemokines

The collected blood was allowed to clot for at least 30 min before separation. The blood containing tubes were then centrifuged for 15 min at 1600 *xg*. Sera were separated from the blood, aliquoted and stored at −70 °C. The sera were analyzed for 14 different proteins including cytokines TNF-α, IL-10, IL-6, IL-12p70, IL-2, IFN-γ, chemokines CXCL10, CCL2, CCL3, CCL4 and apoptotic molecules sTRAIL, sFas, sFasL, MIF. Levels of sTRAIL in undiluted sera were quantified by using a Diaclone solid phase sandwich ELISA kit (#850.770.096; Diaclone, Besancon Cedex, France) according to the manufacturer's protocol. Detection limits were 64 pg/ml. TRAIL absorbancies were read with a Multiskan MS version 4.0 spectrophotometer (Labsystems, Helsinki, Finland) at wavelength 450 nm.

The levels of sFas, sFasL and MIF were measured with a Human sepsis/apoptosis LINCOplex Kit (Linco Research, St. Charles, Missouri, USA), CCL2, CCL3, CCL4 and CXCL10 with a Human cytokine LINCOplex kit (Linco Research) and IL-2, IL-6, IL-10, IL-12p70, IFN-γ and TNF-α with High Sensitivity Human cytokine LINCOplex kit (Linco Research). All the data were collected and analyzed using Bio-Plex suspension array system and Bio-Plex Manager software 4.1 (Bio-Rad laboratories, California, USA). A five-parameter regression formula was used to calculate the sample concentration from the Human sepsis/apoptosis LINCOplex Kit and the High Sensitivity Human cytokine LINCOplex kit, and a four-parameter regression formula was used for the Human cytokine LINCOplex kit. All 96 well plates included samples from all disease subtypes and controls to minimize inter-assay variation. The same batch of monoclonal antibodies for the Bio-Plex Cytokine Assay

Table 1
Clinical characteristics of patients with MS and healthy controls.

Characteristics	CDMS[f]	RRMS	SPMS	PPMS	CIS	HC
Number of patients, baseline[a]	72	33	18	21	17	21
Gender F/M[a]	48/24	24/9	12/6	12/9	14/3	13/8
Age (years)[b]	46 (38–54)	39 (30–44)	52 (44–55)	58 (51–65)	34 (28–42)	42 (28–57)
Time since first symptoms (years)[b]	13.9 (6.7–19.1)	8.2 (4.0–12.7)	19.0 (14.2–25.6)	16.2 (12.2–20.0)	1.0 (0.8–2.2)	–
Age at onset (years)[b]	30 (25–38)	27 (24–34)	29 (24–35)	40 (35–43)	33 (26–39)	–
Disease duration (years)[b]	5.8 (1.3–12.3)	2.8 (1.1–7.1)	13.2 (12.2–23.3)	11.2 (4.5–16.5)	–	–
Progression index[b,c]	0.5 (0.2–1.4)	0.4 (0.2–1.4)	0.5 (0.3–1.9)	0.4 (0.2–0.9)	–	–
EDSS, baseline[b]	3.0 (1.5–6.0)	1.5 (1.0–2.5)	4.5 (3.0–6.1)	5.5 (3.5–6.3)	0 (0.0–0.0)	–
EDSS, 1-year[b]	3.0 (1.5–6.0)	1.5 (1.5–2.6)	6.0 (3.5–6.0)	6.0 (2.8–6.5)	0 (0.0–0.0)	–
Number of relapses, baseline[a,d]						
0	23	7	16	NA	4	–
1	12	11	1	NA	11	–
2–5	16	15	1	NA	2	–
Number of relapses, 1-year[a,e]						
0	34	18	16	NA	15	–
1	10	9	1	NA	2	–
2–5	3	3	0	NA	0	–
Treatment, baseline NT/IFN/GA[a]	49/20/3	11/19/3	17/1/0	21/0/0	17/0/0	–
Treatment, 1-year NT/IFN/GA[a]	45/17/2	11/17/2	17/0/0	17/0/0	14/3/0	–
Duration of the treatments, baseline (months)[b]	16 (7–38)	14 (6–32)	122			

CDMS clinically definite MS group, RRMS relapsing remitting MS, SPMS secondary progressive MS, PPMS primary progressive MS, CIS clinically isolated syndrome, HC healthy controls, EDSS expanded disability status scale, IFN interferon-β, GA glatiramer acetate, NT no treatment.

[a] Number of patients.
[b] Median (25th–75th percentiles).
[c] EDSS/duration of the disease (years).
[d] All relapses during preceding 2 years.
[e] All relapses during preceding year.
[f] Group comprising RRMS, SPMS and PPMS patients.

System was used throughout the experiments; the inter-assay and intra-assay values are reported to be less than 15% by the manufacturer. The percent recovery of standards ranged from 90% to 110% that was used as a detection limit for each protein. The lower detection limits were as follows: 12.2 pg/ml for sFas, sFasL and MIF, 16.0 pg/ml for CXCL10, CCL3 and CCL4, 3.2 pg/ml for CCL2, 0.13 pg/ml for IL-10, TNF-α, IL-6, IL-12p70, IL-2 and IFN-γ.

2.3. MRI: image segmentation and volumetric analysis

All examinations were performed on a 1.5 Tesla MRI Unit (Siemens Avanto, Erlangen, Germany). The MRI protocol for this examination included a T1 weighted header followed by an axial T1- weighted magnetization prepared rapid gradient echo (MP-RAGE), and a T2-weighted turbo spin-echo (TSE), fluid attenuation inversion recovery (FLAIR), magnetization transfer contrasts (MTC), diffusion weighted imaging (DWI), and gadolinium enhanced T1 weighted MP-RAGE sequences. In this study, T1 weighted MP-RAGE, FLAIR and T2-weighted TSE images were used for volumetric analysis. For MP-RAGE, the imaging parameters were as follows: repetition time (TR) = 1160 ms; echo time (TE) = 4.24 ms; inversion time (TI) = 600 ms; slice thickness = 0.9 mm; in-plane resolution = 0.45*0.45 mm. In FLAIR, the following parameters were used: TR = 8500 ms; TE = 100 ms; TI = 2500 ms; slice thickness = 5.0 mm; in-plane resolution = 0.45*0.45 mm. In TSE, the following imaging scheme was used: TR = 750 ms; TE = 115 ms; slice thickness = 3.0 mm; in-plane resolution = 0.90*0.90 mm. Volumetric segmentation of plaques in the brain was performed using semiautomatic software Anatomatic™ operating in a PC/Window 95 environment (Heinonen et al., 1998b, Heinonen et al., 1998a) and the images were analyzed blindly.

2.4. Statistics

Since the immunological data were not normally distributed, the values were tested by non-parametric analyses: the Kruskal–Wallis test for comparison of more than two unpaired groups, the Mann–Whitney *U* Test for comparison of two unpaired groups, and the Wilcoxon test for paired groups. Spearman's correlation coefficient was used to explore the correlation between clinical parameters and the levels on different proteins. Due to the multiple comparisons, the criterion for statistical significance was p-value less than 0.01.

3. Results

3.1. Clinical follow-up data

RRMS patients had a lower EDSS score than the patients with SPMS and PPMS (Table 1). At one-year follow up, the EDSS score had increased in 8 out of 30 (27%) RRMS patients, in 6 out of 17 (35%) SPMS patients, and 6 out of 17 (35%) of patients with PPMS. Two years before enrollment, 7 of the 33 (21%) RRMS patients were relapse-free, 11 (33%) patients had had one relapse and the remaining 15 (45%) patients had had 2 to 3 relapses. Over the one-year follow up, 18 of 30 (60%) RRMS patients were relapse-free, 9 (30%) patients had one relapse and 3 (10%) patients had two to three relapses. Initially, 20 of 72 (28%) CDMS patients were treated with IFN-β and three with glatiramer acetate (GA) (4%). During the follow up, four patients did not respond to treatments and, therefore, two IFN-β and one GA treatments were discontinued and one IFN-β treatment was changed to GA. In addition, two untreated RRMS patients started IFN-β therapy due to active disease.

In 17 CIS patients, the time since the first demyelinating event suggestive of MS ranged from 1 to 8 years (median 1 year). Their EDSS score at the baseline was 0 except for one subject who had a score of 1. Four of the seventeen patients were symptom-free two-years preceding study entry. An elevated IgG index and OCB were found in 10 CIS patients. Over the one-year period, the EDSS score increased in two CIS patients and three of them converted into CDMS.

3.2. The levels of cytokines, chemokines and apoptotic molecules at baseline

The baseline levels of chemokines, inflammatory cytokines, and apoptotic molecules in MS patients and controls are shown in Table 2. Decreased MIF and elevated TNF-α levels were detected in the CDMS group (including all MS patients except those with CIS) when compared to controls (MIF: 195.0 vs. 378.5 pg/ml, $p<0.001$; TNF-α: 4.7 vs. 3.7 pg/ml, $p=0.005$). Comparisons between respective subtypes and controls revealed a decreased MIF in RRMS (138.2 vs. 378.5 pg/ml, $p<0.001$) and increased sFas, TNF-α and CCL2 levels in PPMS (sFas: 6787.6 vs. 4824.9 pg/ml, $p=0.01$; TNF-α: 6.4 vs. 3.7 pg/ml, $p<0.001$; CCL2: 288.4 vs. 152.8 pg/ml, $p=0.005$, Table 2). Further comparison between MS subtypes revealed higher MIF, TNF-α and sFas in PPMS than in RRMS (MIF: 290.7 vs. 138.2 pg/ml, $p=0.001$; TNF-α: 6.4 vs. 4.5 pg/ml, $p=0.010$; sFas: 6787.6 vs. 4612.8 pg/ml, $p=0.002$). The levels of

Table 2
The levels (pg/ml) of soluble molecules in different types of MS and controls [median (25th–75th percentiles)].

Molecule	CDMS N = 72	RRMS N = 33	SPMS N = 18	PPMS N = 21	CIS N = 17	HC N = 21
sFas	5394.3 (4343.7–6833.5)	4612.8 (4058.0–5940.7)[d]	5572.4 (4388.3–6845.4)	6787.6 (5393.9–7709.2)[c,d]	4318.8 (3320.0–5697.9)	4824.9 (3405.1–5638.1)[c]
sFasL	94.1 (67.6–168.6)	90.6 (58.1–161.6)	96.2 (72.8–153.6)	111.2 (71.5–191.9)	83.6 (67.1–174.6)	80.4 (56.6–129.1)
MIF	195.0 (100.2–298.0)[a]	138.2 (50.0–229.4)[b,d]	225.8 (134.0–317.3)	290.7 (189.3–449.3)[d]	167.3 (97.8–268.2)	378.5 (236.4–535.9)[a,b]
sTRAIL	845.2 (645.2–1603.0)	765.9 (577.2–1728.9)	785.5 (587.2–1106.0)	1032.0 (762.8–2036.1)	902.3(593.3–1170.5)	738.7 (523.2–2260.8)
CXCL10	98.9 (66.1–154.7)	95.7 (68.1–155.6)	90.5 (62.8–152.6)	106.9 (74.7–212.9)	65.9 (53.6–178.2)	106.9 (55.1–130.7)
CCL2	218.3 (154.7–371.4)	177.0 (140.4–271.8)	218.8 (157.0–331.0)	288.4 (201.3–461.6)[c]	248.3 (165.7–332.0)	152.8 (122.6–273.1)[c]
CCL3	50.9 (22.4–99.3)	37.3 (21.6–96.3)	55.7 (19.2–121.4)	45.4 (27.0–164.7)	72.6 (32.0–186.1)	51.4 (27.7–122.8)
CCL4	160.6 (24.6–835.5)	126.6 (20.4–668.0)	140.2 (17.0–873.8)	290.7 (39.8–1054.6)	365.1 (144.0–1188.5)	289.1 (16.8–725.0)
IL-10	3.2 (1.6–7.1)	2.9 (0.6–5.7)	3.4 (1.8–7.7)	3.6 (2.2–9.2)	4.8 (1.2–25.9)	2.3 (0.2–6.0)
TNF-α	4.7 (3.7–7.3)[a]	4.5 (3.4–6.5)[d]	4.1 (3.3–5.8)	6.4 (4.4–8.9)[c,d]	4.6 (3.5–6.6)	3.7 (2.4–4.6)[a,c]
IL-6	7.0 (2.1–18.4)	4.9 (2.0–21.5)	7.1 (3.2–13.0)	8.8 (1.6–26.7)	3.6 (1.4–17.9)	4.1 (2.0–9.3)
IL-12p70	0.6 (0.1–3.0)	0.4 (0.1–1.4)	1.3 (0.4–5.4)	0.8 (0.1–4.5)	1.7 (0.3–9.1)	1.6 (0.1–7.9)
IFN-γ	0.6 (0.3–3.5)	0.3 (0.3–3.0)	1.5 (0.3–4.1)	0.6 (0.3–3.3)	2.8 (0.3–25.5)	0.5 (0.3–7.5)
IL-2	0.6 (0.2–3.6)	1.2 (0.2–3.6)	0.6 (0.2–5.2)	0.4 (0.2–2.4)	1.5 (0.5–6.2)	2.1 (0.2–6.1)

CDMS clinically definite MS (RRMS, SPMS and PPMS), RRMS relapsing remitting MS, SPMS secondary progressive MS, PPMS primary progressive MS, CIS clinically isolated syndrome, HC healthy controls.

[a] Comparison between CDMS and HC group, the Mann–Whitney *U* Test $p<0.01$.
[b] Comparison between RRMS and HC group, the Mann–Whitney *U* Test $p<0.01$.
[c] Comparison between PPMS and HC group, the Mann–Whitney *U* Test $p<0.01$.
[d] Comparison between the PPMS and RRMS group, the Mann–Whitney *U* Test $p<0.01$.

chemokines CCL3, CCL4, CXCL10, cytokines IL-2, IL-10, IL-6, IL-12p70 IFN-γ or the apoptotic molecules sFasL and sTRAIL did not differ between the groups ($p>0.01$). Nineteen IFN-β treated RRMS patients had increased levels of CXCL10 compared to 11 untreated patients (median 56.0 vs. 112.6 pg/ml, $p=0.001$).

3.3. Relation of immunological profiles to clinical characteristics

The association of baseline immunological findings with disease activity or progression was studied by correlating the levels of different molecules with the patients' EDSS scores, progression index and disease activity at baseline and after one year. In the CDMS group, only baseline MIF ($r=0.39$, $p=0.001$, $n=72$) correlated with the EDSS score (Fig. 1A), but within the subtypes statistically significant correlations were not found. Over one year, the EDSS score had increased in 20 out of 64 CDMS patients. To further analyze the association between immune molecules and disease progression over the 1 year, CDMS patients were divided into two groups: patients with disability progression (EDSS score increased more than 0.5, $n=20$) and those with stable EDSS (EDSS score unchanged, $n=44$). In the worsening patients, the levels of sFas (6353.4 0 vs. 4993.2 pg/ml, $p=0.019$) and MIF (272.4 vs. 158.3 pg/ml, $p=0.020$) were higher than in the stable group (Fig. 1B and C), but no associations were found between the molecules and the progression index or disease activity as expressed by the number of relapses.

3.4. Volumes of T1 and FLAIR lesions

At baseline, the volumes of MS plaques were determined in the 89 patients (33 RRMS, 18 SPMS, 21 PPMS, 17 CIS), and over 1 year in 81 (29 RRMS, 17 SPMS, 17 PPMS, 17 CIS) of them. The baseline median volumes for T1 hypointense and FLAIR-weighted plaques were 1.6 cm^3 and 4.9 cm^3 in the CDMS group respectively (Table 3). The T1 lesions represent axonal loss and appear to contribute to neurological disability and the FLAIR lesions represent a total estimate of both the active and chronic inflammatory plaques especially in the supratentorial region (Tomassini and Palace, 2009). The volumes of T1 and FLAIR lesions tended to be lowest in PPMS, although the differences between the subtypes did not reach statistical significance ($p>0.01$). In CIS patients, the volumes of T1- and FLAIR-lesions were smaller than in the CDMS group (T1: 0.3 vs. 1.6 cm^3, $p<0.001$ and FLAIR: 1.1 vs. 4.9 cm^3, $p<0.001$). During the one-year follow up, the most marked change (Δ) in the T1- lesion volume was seen in SPMS ($\Delta T1=1.7$ cm^3, $p=0.018$), while the volumes of FLAIR-weighted plaques increased in all MS subtypes (ΔFLAIR, RRMS: 2.8 cm^3, $p=0.002$, SPMS: 5.3 cm^3, $p=0.007$, PPMS: 3.2 cm^3, $p=0.002$). No changes were seen in the volumes of FLAIR lesion in CIS ($\Delta FLAIR=0.5$ cm^3, $p=0.02$).

3.5. Relation of immunological findings to volumetric MRI measurements

To study the association between immunological molecules and MRI measurements at the baseline, the levels of chemokines, cytokines and apoptotic proteins were correlated to volumes of T1 and FLAIR lesions at baseline. In the CDMS group, no associations were found between the volumes of T1 or FLAIR lesions and soluble molecules. However, in the combined group of patients with RRMS and SPMS, significant correlation was detected between the levels of IL-6 and the volumes of FLAIR lesions ($r=0.368$ $p=0.009$, $n=51$) (Fig. 2A).

In spite of the increase in FLAIR lesion volumes in the CDMS group over one year, correlations between immunological and MRI findings were not detected. To further evaluate the association between immunological responses and MRI changes consistent with neurodegeneration, the patients with RRMS and SPMS were divided into two groups: those with increased volumes of T1 over 1 year (progressed, $\Delta T1\geq 1.0$ cm^3, $n=19$) and stable ones (stable, $\Delta T1<1.0$, $n=27$).

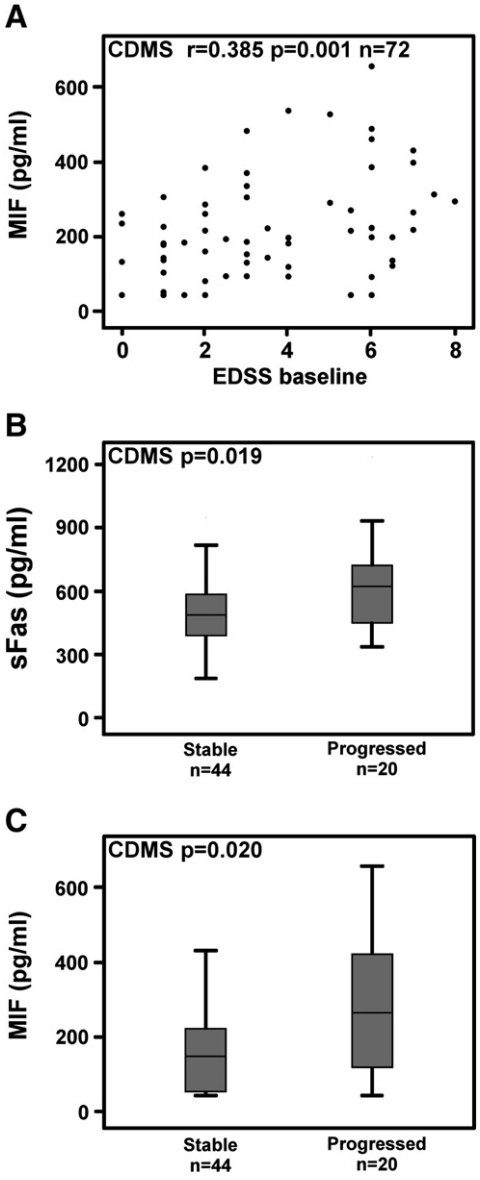

Fig. 1. Association between EDSS score and the levels of MIF and sFas in CDMS group. (A) correlations between baseline EDSS and the levels of MIF in clinically definite MS (CDMS) group. (B–C) increased levels of sFas and MIF were found in patients with disability progression in CDMS group. CDMS patients were divided into two groups: patients with disability progression (EDSS score increased more than 0.5, $n=20$) and those without progression of disability (EDSS score unchanged, $n=44$) over 1 year follow-up. The length of the box represents the interquartile range within which 50% of the values were located. The line through the middle of each box represents the median. The error bars show the minimum and maximum values (range).

Upregulated levels of sFas were found in patients with increasing T1 lesion volumes (5973.8 vs. 4408.1 pg/ml, $p=0.010$) in comparison with the stable group (Fig. 2B).

4. Discussion

In this study, the profiles of major cytokines, chemokines, and apoptotic molecules that are thought to be involved in the pathophysiology of MS were correlated with clinical and MRI follow-up characteristics with the aim of identifying the biomarkers of disease activity and progression in MS. Higher levels of sFas, TNF-α and CCL2 in sera of PPMS patients at baseline in comparison to healthy controls indicate the presence of inflammatory activity in this subtype where neurodegenerative changes were previously considered to

Table 3
The volumes of T1 and FLAIR lesions in MS subtypes [median (25th–75th percentiles)].

MRI volumes	CDMS N = 72	RRMS N = 33	SPMS N = 18	PPMS N = 21	CIS N = 17
Number of patients, baseline/1-year	72/64	33/29	18/17	21/17	17/17
T1 (cm^3)					
Baseline	1.6 (0.5–4.2)*	1.0 (0.4–3.2)	2.9 (1.5–8.4)	1.3 (0.4–5.3)	0.3(0.1–1.2)*
1-year	2.6 (0.8–6.7)	1.9 (0.7–4.8)	5.8 (2.6–8.6)	0.8 (0.6–3.1)	0.5 (0.2–0.9)
Δ T1**	0.4 (−0.2–1.6)	0.3(−0.2–1.2)	1.7 (0.0–3.4)***	0.2 (−0.5–0.6)	0.0 (−0.3–0.4)
FLAIR (cm^3)					
Baseline	4.9 (2.0–15.5)*	3.4 (1.7–11.4)	13.4 (4.1–20.1)	4.6 (1.8–14.5)	1.1 (0.4–2.9)*
1-year	11.2 (3.1–22.1)	8.7 (2.5–17.5)	19.2 (10.4–27.6)	5.4 (3.3–12.7)	2.3 (1.0–6.0)
Δ FLAIR**	3.6 (0.8–9.0)***	2.8 (0.7–10.0)***	5.3 (0.8–14.6)***	3.2 (1.2–6.3)***	0.5 (0.4–1.9)

CDMS clinically definite MS group (RRMS, SPMS and PPMS), RRMS relapsing remitting MS, SPMS secondary progressive MS, PPMS primary progressive MS, CIS clinically isolated syndrome, FLAIR fluid attenuation inversion recovery.
* Comparison between CIS and CDMS group, the Mann–Whitney *U* Test $p<0.01$.
** Changes of MRI volumes from baseline to 1-year follow-up.
*** Comparison between the MRI volumes from the baseline to 1-year follow-up, the Wilcoxon test $p<0.01$.

prevail over inflammation (Frischer et al., 2009, Bradl and Lassmann, 2009, Lassmann et al., 2007). In line with this concept are the results from the clinical trial on the efficacy of Rituximab in PPMS indicating delayed time to confirm disease progression in Rituximab-treated patients with disease activity on MRI (Hawker et al., 2009). The involvement of the Fas-FasL system in the pathogenesis of MS has been suggested by several authors (Zipp, 2000, Pender, 2007). In particular, death receptor Fas defects have been reported in the T lymphocytes of MS patients, and especially in the chronic progressive subtype. These defects were considered to promote the survival of autoreactive T cells (Comi et al., 2000, Sharief, 2000). The soluble forms of Fas have been shown to inhibit apoptotic events in T cells (Aktas et al., 2006). Our observation of increased sFas in PPMS is in line with previous data thus favoring the concept of dysregulated Fas-mediated apoptosis in MS (Ciusani et al., 1998, Boylan et al., 2001). In addition, TNF-α and CCL2 have been identified in active MS lesions (Selmaj et al., 1991) and increased levels of TNF-α in sera and CSF together with intrathecal synthesis of CCL2 have been reported in PPMS (Sharief and Hentges, 1991, Hohnoki et al., 1998).TNF-α is a well-known trigger of the apoptosis of oligodendrocytes (Watzlawik et al., 2010), while CCL2 is involved in the recruitment of monocytes and T cells from blood to brain parenchyma (Mahad and Ransohoff, 2003). Taken together, our findings of increased levels of sFas, TNF-α and CCL2 in sera of PPMS patients together with earlier evidence are consistent with the involvement of these molecules in the pathogenesis of this subtype.

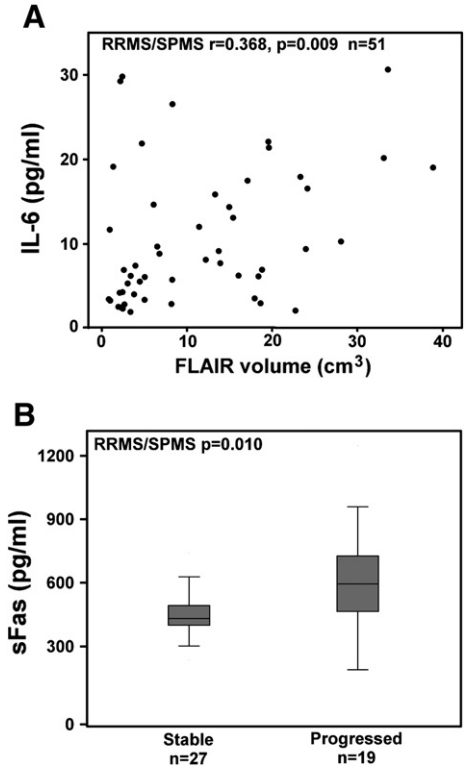

Fig. 2. Association between the volumes of lesions and the levels of IL-6 and sFas in group comprising RRMS and SPMS patients. (A) statistically significant correlation was found between the volume of FLAIR lesion and the levels of IL-6 in RRMS and SPMS patients. (B) upregulated levels of sFas were found in those RRMS and SPMS patient whose T1 lesion volumes increased in comparison to stable group of patients. RRMS and SPMS patients were divided in to two groups: those who increased the volumes of T1 from baseline to one-year follow-up (progressed, n = 19) and those with unchanged T1 volumes (stable, n = 27). The length of the box represents the interquartile range within which 50% of the values were located. The line through the middle of each box represents the median. The error bars show the minimum and maximum values (range).

Lower levels of serum MIF in RRMS as compared to PPMS and controls are in contrast to earlier data that have shown upregulated MIF in blood and CSF during MS relapse (Niino et al., 2000, Rinta et al., 2008). Calandra et al. have shown that MIF is released from cytoplasmic stores following stimulation by endotoxin, exotoxin and cytokines such as TNF and IFN-γ and it can act in an auto and paracrine fashion to stimulate its own synthesis and the synthesis of other proinflammatory mediators (Calandra et al., 1994). Studies using experimental autoimmune encephalomyelitis (EAE) have shown that MIF induces the accumulation of encephalitogenic T cells in the CNS thus facilitating the development of EAE (Denkinger et al., 2003, Kithcart et al., 2010). More recently MIF was identified as a non-cognate ligand of the chemokine receptors CXCR2 and CXCR4 (Bernhagen et al., 2007). Taken together, *these observations suggest that MIF is associated with disease activity and can potentiate effects of other proinflammatory stimuli. Decreased levels of MIF found in our RRMS most likely are explained by clinically stable disease,* although their FLAIR lesions volumes increased over 1 year indicating the presence of persistent inflammation in the CNS. The discrepancy between these clinical and radiological observations are most likely explained by the fact that MRI is extremely sensitive for detecting new inflammatory activity in the CNS that is often clinically silent (Barkhof, 2002). Correlation analyses between the immunological findings and MRI measurements showed a positive correlation only between the serum IL-6 and the baseline volumes of FLAIR lesions in the RRMS and SPMS groups indicating the role of IL-6 in neuroinflammation. IL-6 expression is upregulated under inflammatory conditions and it has a variety of immunoregulatory functions including differentiation of Th17 cells (Afzali et al., 2010).

During the follow up, the levels of sFas increased in those patients whose EDSS score and T1 lesion volumes increased. T1 lesions represent axonal loss that contributes to the neurological disability in MS (Tomassini and Palace, 2009). Evidence on the predictive value of Fas in the evaluation of disease course has been contradictory (Zipp et al., 1998, Lopatinskaya et al., 2006). One study reported association between the increased levels of sFas in sera of patients with short-term disease progression (Zipp et al., 1998), while a recent 10-year follow-up study reported an association between increased blood mRNA expression with favorable disease course in RRMS and SPMS (Lopatinskaya et al., 2006). Our observations of increased sFas in worsening patients support the concept of sFas promoting auto-reactive T cell survival that in turn is likely to facilitate neurodegenerative events. Likewise, the positive correlation between MIF and EDSS scores in the MS group suggests the involvement of MIF in the neurodegeneration that is further supported by the finding of increased MIF in patients with disability progression over 1 year.

In summary, according to our data, Fas and MIF may be considered candidate biomarkers of disease progression in MS. However, although TNF-α and CCL2 seem to reflect MS-related inflammatory responses, especially in PPMS, their role as biomarkers of clinical disease activity remains to be evaluated in a long-term study involving a larger patient cohort.

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Is the modulatory effect of pregnancy in multiple sclerosis associated with changes in blood apoptotic molecules?

Rinta S, Airas L, Elovaara I. Is the modulatory effect of pregnancy in multiple sclerosis associated with changes in blood apoptotic molecules? Acta Neurol Scand: 2010: 122: 168–174.
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Objective – We examined whether the modulatory effect of pregnancy on multiple sclerosis (MS) is associated with changes in the apoptotic molecules in sera. *Subjects and methods* – The serum levels of tumor necrosis factor-related apoptosis-inducing ligand (sTRAIL), sFas, Fas ligand (sFasL) and macrophage migration inhibitory factor were analyzed from 19 MS patients and 14 controls during late pregnancy and post-partum. The obtained results were related to disease activity and the progression of MS. *Results* – Disease activity decreased during pregnancy. The levels of sTRAIL and sFasL increased from late pregnancy to post-partum situation in both MS patients and controls, but in MS patients the changes in the levels of sTRAIL from late pregnancy to post-partum were smaller than in controls. *Conclusions* – Post-partum upregulation of TRAIL and FasL seems to be caused by physiologic reactivation of the mother's immune system after pregnancy. An increased risk of relapses in MS post-partum may be associated with changes in the immunomodulatory potential of these apoptotic molecules.

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Key words: Multiple Sclerosis; Pregnancy; TNF-related apoptosis-inducing ligand; Fas; Fas ligand; macrophage migration inhibitory factor

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Introduction

The annualized relapse rate (ARR) of multiple sclerosis (MS) declines during the third trimester of pregnancy, but increases again during the first 3 months post-partum before returning to the prepregnancy rate (1). This effect of pregnancy on MS disease activity has been explained by changes in the mother's endocrinal and immune system and is characterized by an increase in the levels of the sex hormones estrogen and progesterone together with a shift of maternal immune responses from a prevailing T-helper cell (Th)1 responses to a Th2-type response. An increase in regulatory cytokines interleukin (IL)-4 and IL-10 together with downregulation of proinflammatory cytokines interferon (IFN)-γ and IL-2 in women at late pregnancy (2–6) has been reported. Based on *in vitro* evidence, increased levels of estrogens can inhibit expression of proinflammatory cytokines IL-6, IL-8 and tumor necrosis factor (TNF)-α and stimulate expression of regulatory cytokines IL-10, IL-4 and transforming growth factor (TGF)-β (7). It is proposed that a shift in maternal immune responses from a prevailing Th1 response to a Th2-type response is necessary for protecting the developing fetus (4, 8). Interestingly, a decrease of circulating natural killer (NK) cells and an increase of regulatory T cells during pregnancy has been associated with reduced MS disease activity (9, 10). Moreover, an increase in circulating regulatory CD56 (bright) NK cells and a decrease of circulating cytotoxic CD56 (dim) NK cells in late pregnancy was considered to be associated with inhibition of MS activity during pregnancy (11).

Although understanding of the amelioration of MS during pregnancy has improved recently, the exact mechanism of this phenomenon still remains obscure (12). Upregulation of soluble apoptotic molecules including TNF-related apoptosis-inducing ligand (sTRAIL), Fas ligand (sFasL) and

macrophage migration inhibitory factor (MIF) has been related to immune activation in MS (13), but it is not known whether changes in the levels of these proteins are associated with modulation of MS during pregnancy. To answer this question, we analyzed the levels of sTRAIL, sFas, sFasL and MIF in sera of pregnant women with MS at late pregnancy and post-partum and related the obtained data to ARR and Expanded Disability Status Scale (EDSS) score.

Patients and methods

Patients

The study included 19 pregnant patients with relapsing remitting MS (RRMS) and 14 pregnant healthy controls (Table 1). The mean (±SE) age of RRMS patients at early pregnancy was 30.1 ± 0.7 years and in controls 30.1 ± 1.1 years. Patient recruitment and neurological examinations were performed as reported previously (11). Patients underwent neurological examination at 10–12 weeks of pregnancy (early pregnancy), 26–28 weeks of gestation (late pregnancy) and 6 months post-partum. Clinical characteristics (mean ± SE) of the patients at the beginning of the study are given in Table 1. At the beginning of the study the duration of MS from the time of diagnosis was 5.7 ± 0.9 years (range 0–13), the total number of relapses was 4.1 ± 0.7 (range 0–12 relapses) and EDSS was 1.1 ± 0.2. Before pregnancy six patients were treated with IFN-β (Rebif®, Merck Serono S.A., Modugno, Italy, $n = 2$; Betaferon®, Bayer Schering pharma AG, Berlin, Germany, $n = 3$; and Avonex, Biogen Idec BV, Hoofddorp, Netherlands, $n = 1$) and three patients with glatiramer acetate (Copaxone®, Teva Pharmaceuticals Europe B.V., Utrecht, Netherlands). In most of the patients the medication was discontinued before pregnancy, and only three patients continued on immunomodulatory treatment until the first month of pregnancy.

Table 1 Patients' demographics at the onset of the study

Characteristics	MS ($n = 19$)	Controls ($n = 14$)
Age (years)[a]	30.1 ± 0.7	30.1 ± 1.1
Duration of disease (years)[a]	5.7 ± 0.9	NA
Number of relapses[a,b]	4.1 ± 0.7	NA
EDSS at initiation[a,c]	1.1 ± 0.2	NA
DMT before pregnancy[d]	9	NA
Rebif®	3	NA
Betaferon®	3	NA
Copaxone®	3	NA
DMT during 1–6 months PP[d]	9	NA
Rebif®[e]	5	NA
Betaferon®	1	NA
Avonex®	1	
Copaxone®[e]	2	NA
Gestational weeks[a]	40.1 ± 0.32	NA
Breastfeeding[d]	17	NA
Blood sample collection[d]	19	14
During late pregnancy[d]	19	14
During 3/6 months PP[d]	16/3	14

[a]Mean ± SE.

[b]Total number of relapses experienced before the onset of the study.

[c]EDSS for pregnant patients at 10–12 gestational weeks.

[d]Number of patients.

[e]Rebif was initiated in two patients and Copaxone was initiated in one patient before post-partum sampling.

MS, multiple sclerosis; EDSS, Expanded Disability Status Scale; DMT, disease-modifying therapy; PP, post-partum; NA, not available.

Blood sample collection

The blood from MS patients was drawn during late pregnancy and 3 ($n = 16$) or 6 ($n = 3$) months after delivery, whereas the sera from healthy pregnant women ($n = 14$) were collected during late pregnancy and 3 months post-partum.

The cytokine determinations

The levels of sFas, sFasL and MIF in sera were measured simultaneously with a Human Apoptosis Lincoplex Kit (Linco Research, St Charles, IL, USA) and sTRAIL levels were measured using an ELISA kit (Diaclone, Besancon Cedex, France) as previously reported (13).

Statistical analyses

Statistical analyses were performed using SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL, USA). The numbers of relapses a year before pregnancy, during pregnancy and 6 months post-partum follow-up were converted to a relapse rate per patient per year (ARR). The changes of different parameters (ΔARR, ΔEDSS, ΔTRAIL, ΔFas, ΔFasL and ΔMIF) at different time points were also studied. Comparisons between pre- and post-partum ARR, EDSS and levels of these molecules were performed using the Wilcoxon test, and comparison between MS patients and controls, and active and stable patients by the Mann–Whitney test. A P-value less than 0.05 was considered statistically significant. Correlations between variables were tested by two-tailed non-parametric Spearman correlation analysis. Owing to multiple correlation testing, a P-value of less than 0.01 was considered significant.

Results

Activity of MS during and after pregnancy

Comparison between the means (±SE) of ARR before and during pregnancy revealed a decrease in ARR from prepregnancy situation to pregnancy (0.9 ± 0.2 to 0.4 ± 0.2 relapses/woman/year; $P = 0.004$; Table 2), but the change between the ARRs before pregnancy and post-partum was not significant (0.9 ± 0.2 vs 1.5 ± 0.3 relapses/woman/year; $P = 0.10$). Comparison between ARRs during and after pregnancy showed a statistically significant increase in ARR after pregnancy (0.4 ± 0.2 vs 1.5 ± 0.3 relapses/woman/-year, $P = 0.01$). Fifteen out of the 19 MS patients were relapse-free during pregnancy, 3 patients had a relapse during the first or second trimester and only 1 patient experienced a relapse during the third trimester. During the 6-month post-partum follow-up, 7 patients remained relapse-free and 12 patients had 1–3 relapses. The majority (8 of 12) of the post-partum relapses occurred during the first 3 months after pregnancy.

The mean (±SE) EDSS tended to increase during the 6-months post-partum follow-up, but the differences between EDSS at different timings were not statistically significant (1.1 ± 0.2 vs 1.2 ± 0.2 vs 1.5 ± 0.2; $P > 0.05$; Table 2). Four patients were prescribed immunomodulatory treatment during the first 3 months post-partum, and in 5 patients the treatment was started between 3 and 6 months post-partum.

The levels of apoptosis-related molecules during late pregnancy and after pregnancy

The levels of sTRAIL, sFasL, sFas and MIF in serum samples obtained pre- and post-partum from 19 MS patients and 14 controls are shown in Fig. 1. A significant increase in the levels (mean ± SE) of sTRAIL and sFasL was seen from pre- to post-partum periods in both patients with MS (sTRAIL: 0.38 ± 0.07 vs 0.72 ± 0.11 ng/ml, $P = 0.002$ and sFasL: 0.30 ± 0.11 vs 0.35 ± 0.12 ng/ml, $P = 0.02$) and their controls (sTRAIL: 0.76 ± 0.13 vs 1.51 ± 0.25 ng/ml, $P = 0.004$ and sFasL: 0.16 ± 0.04 vs 0.25 ± 0.05 ng/ml, $P = 0.003$). Moreover, serum sTRAIL levels were lower in patients with MS than in healthy controls both in the pre- and post-partum situations (sTRAIL prepartum: MS 0.38 ± 0.07 vs control 0.76 ± 0.13 ng/ml, $P = 0.0005$ and sTRAIL post-partum: MS 0.72 ± 0.11 vs control 1.51 ± 0.25 ng/ml, $P = 0.002$). The changes (Δ) in the levels of sTRAIL (-0.33 ± 0.11 vs -0.75 ± 0.19 ng/ml, $P = 0.05$) from late pregnancy to post-partum were smaller in MS patients than in controls (Fig. 2). No changes were seen in the levels of sFasL between MS and controls (-0.05 ± 0.02 vs -0.09 ± 0.08 ng/ml, $P = 0.161$).

Over the pre- to post-partum periods the levels of sFas and MIF tended to decrease both in MS patients (sFas: 4.57 ± 0.29 vs 3.96 ± 0.40 ng/ml, $P = 0.08$ and MIF: 0.96 ± 0.19 vs 0.72 ± 0.15 ng/ml, $P = 0.12$) and their controls (sFas: 4.88 ± 0.19 vs 4.19 ± 0.20 ng/ml, $P = 0.03$ and MIF: 1.19 ± 0.40 vs 0.51 ± 0.10 ng/ml, $P = 0.06$), but the difference between Δ values was not statistically significant in these groups (Fig. 2; ΔsFas: 0.61 ± 0.32 vs 1.19 ± 0.56 ng/ml, $P = 0.560$ and ΔMIF: 0.24 ± 0.16 vs 0.67 ± 0.40 ng/ml, $P = 0.500$).

Comparisons between the levels of apoptotic proteins in pre- and post-partum samples were also performed in the subgroup of 15 patients in whom disease-modifying therapy was not initiated before post-partum sampling (Table 1). The analyses revealed upregulation of sTRAIL (0.33 ± 0.04 vs 0.54 ± 0.30 ng/ml, $P = 0.001$) and sFasL (0.32 ± 0.12 vs 0.38 ± 0.13 ng/ml, $P = 0.02$) after delivery. The change (Δ) in TRAIL levels from late pregnancy to post-partum was smaller in untreated MS patients than in controls (-0.20 ± 0.05 vs -0.75 ± 0.19 ng/ml, $P = 0.012$), but the differences between the ΔsFas (0.56 ± 0.35 vs 1.19 ± 0.56 ng/ml, $P = 0.484$), ΔsFasL (-0.05 ± 0.02 vs -0.09 ± 0.02 ng/ml, $P = 0.154$) and ΔMIF (0.32 ± 0.15 vs 0.67 ± 0.40 ng/ml,

Table 2 Multiple sclerosis patients' disease activity and neurologic disability before, during and after pregnancy

	Before pregnancy	During pregnancy	Post-partum	P1	P2	P3
Annualized relapse rate[a]	0.9 + 0.2	0.4 ± 0.2	1.5 ± 0.3	0.004	0.100	0.010
EDSS[a]	1.1 ± 0.2[b]	1.2 ± 0.2	1.5 ± 0.2	0.236	0.253	0.100

[a]Mean ± SE.

[b]EDSS for pregnant patients at 10–12 gestational weeks.

P1 compared between before and during pregnancies; P2 compared between before and after pregnancies; P3 compared between during and after pregnancies; EDSS, Expanded Disability Status Scale.

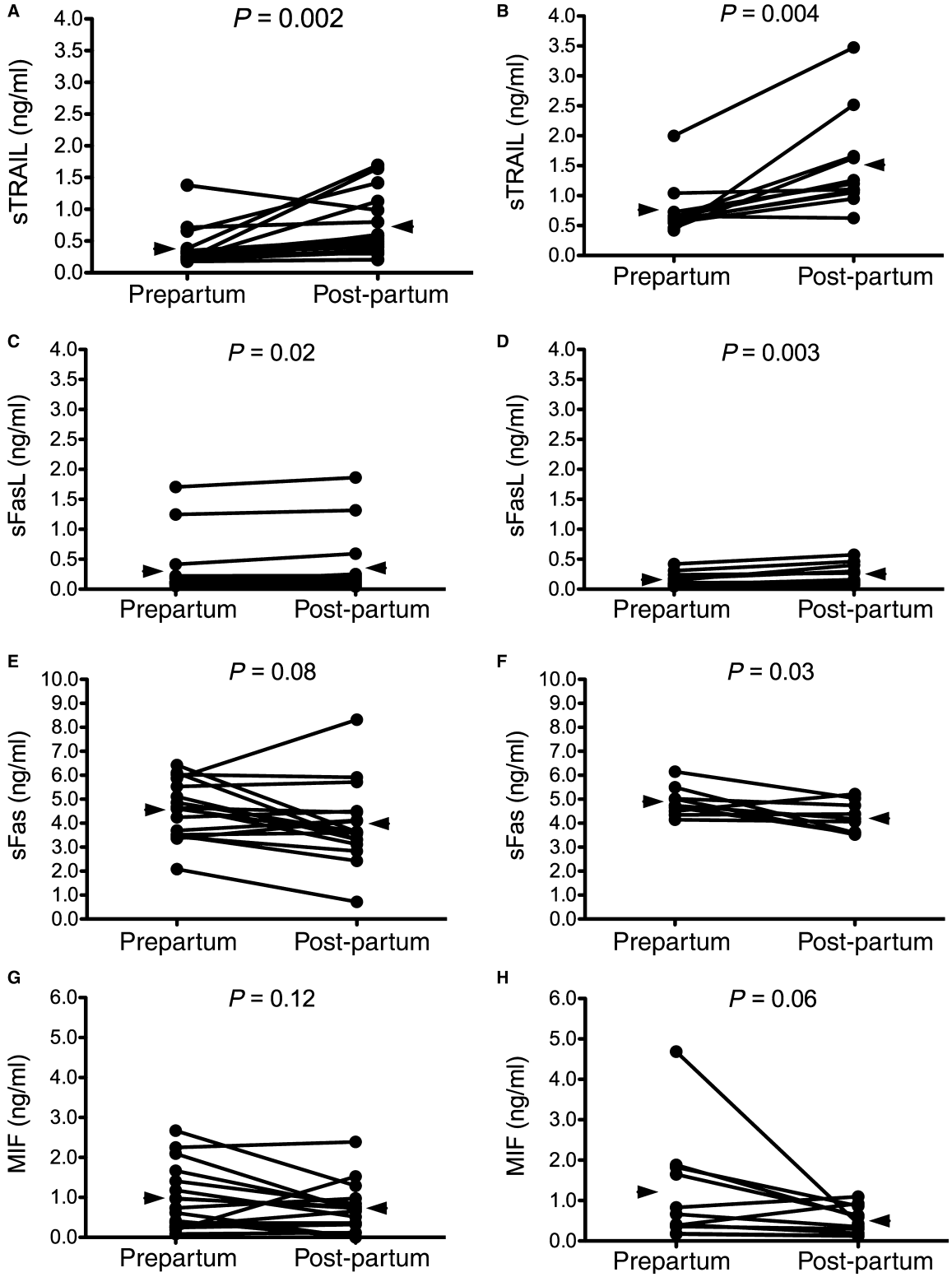

Figure 1. The levels (ng/ml) of tumor necrosis factor-related apoptosis-inducing ligand (A, B), Fas ligand (C, D), sFas (E, F) and macrophage migration inhibitory factor (G, H) during late pregnancy (prepartum) and after delivery (post-partum) in multiple sclerosis patients (A, C, E and G) and healthy controls (B, D, F and H). The levels of molecules are shown for each donor. The arrowhead indicates the mean value.

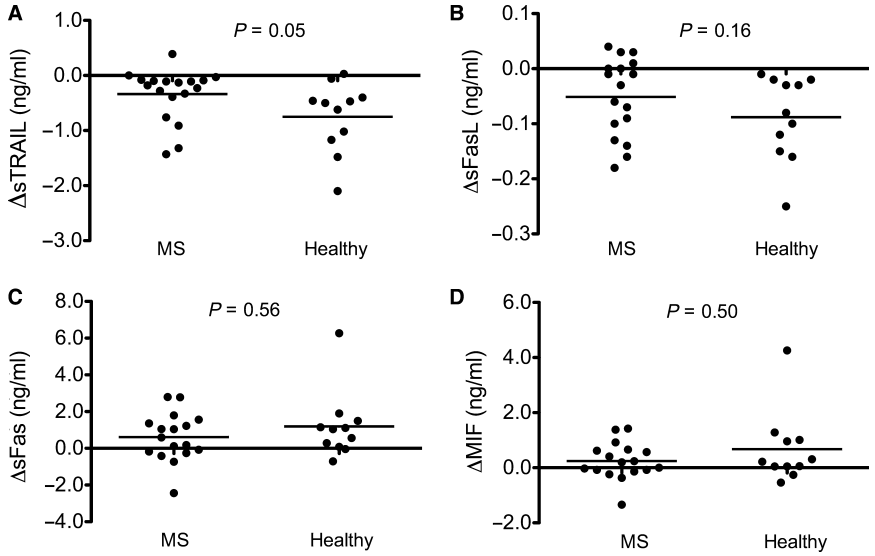

Figure 2. The changes (Δ) in the levels of tumor necrosis factor-related apoptosis-inducing ligand (A), Fas ligand (B), sFas (C) and macrophage migration inhibitory factor (D) seen from late pregnancy to post-partum in multiple sclerosis patients and controls (ng/ml). The levels of molecules are shown for each donor. Horizontal lines indicate the mean values.

$P = 0.586$) in these groups were not significant. IFN-β ($n = 3$) and glatiramer acetate ($n = 1$) treatment was started in the remaining four patients.

Relationship between disease activity and apoptotic proteins

No correlations were found between the levels of serum apoptotic molecules and ARRs or EDSS ($P > 0.01$). Likewise, the changes (Δ) in the levels of apoptotic proteins from late pregnancy to post-partum did not correlate with the changes of ARRs or EDSS ($P > 0.01$).

To further explore the relationship between quantitative changes in the apoptotic molecules and activity of MS, the patients were divided into an active group ($n = 11$, up to three relapses after pregnancy; 1.36 ± 0.15, mean ± SE) and a stable group ($n = 7$, no relapses). Comparison between these groups showed that the patients with active MS had smaller ΔFasL from pregnancy to post-partum than the patients with inactive disease (-0.02 ± 0.06 vs -0.09 ± 0.07 ng/ml, $P = 0.04$). The post-partum levels of sTRAIL ($P = 0.596$), sFas ($P = 0.69$), sFasL ($P = 0.32$) and MIF ($P = 0.67$) in active and stable MS patients were of the same magnitude.

Discussion

In spite of the recent progress in the understanding of the modulatory effect of pregnancy on MS, very little is known about the detailed molecular mechanisms of this phenomenon (12). Previously, it has been hypothesized that failure of activation-induced cell death (apoptosis) of autoreactive T cells is a major pathogenetic mechanism in MS (14). Detection of impaired apoptosis of lymphocytes or T cells obtained from MS patients (15–18) is consistent with this hypothesis. Furthermore, recent studies have shown downregulation of pro-apoptotic molecules in lymphocytes obtained from patients with active MS, suggesting abnormalities in the apoptotic cell death of lymphocytes in MS (19–21). In the present study we analyzed whether the beneficial effect of pregnancy on MS is associated with quantitative changes of apoptotic molecules in serum.

The levels of serum sTRAIL and sFasL in MS patients increased post-partum. Earlier we reported an association between elevated sTRAIL and disease activity in RRMS (13). TRAIL is a member of the TNF superfamily that is expressed in membrane-bound and soluble forms from lymphocytes and monocytes in an activation-dependent manner (22). It has been shown that sTRAIL inhibits proliferation of activated T cells (23, 24) and inhibition of TRAIL outside the central nervous system (CNS) has been shown to worsen Experimental Autoimmune Encephalomyelitis (EAE) (25). However, in the CNS, TRAIL mediates apoptosis of brain cells (26–28). In MS, TRAIL has been shown to reflect bioactivity of IFN-β (29). Post-partum upregulation of sTRAIL in both MS patients and controls may be associated with physiological immune activation known to occur after pregnancy and may reflect a tendency of the immune system to control inflammatory responses. The smaller increase of sTRAIL from late pregnancy to post-partum in MS com-

pared with controls, detected by us, may be a factor associated with activation of MS after delivery.

A significant increase in the levels of sFasL was seen from pre- to post-partum periods in both patients with MS and their controls. Fas receptor (CD95) and FasL (CD154) participate in the apoptotic signaling pathway in the activated T cells (30). Membrane-bound forms of Fas and FasL are able to induce apoptosis of T cells, but soluble forms, in contrast, inhibit apoptotic events (31, 32). The upregulation of sFasL after pregnancy in both MS patients and controls seen in this study may be associated with increased immune activation. This is in accordance with our data showing increased levels of sFasL during MS relapses (13) and results by other investigators reporting increased expression of FasL mRNA in blood cells during lesional activity (33). The similar levels of sFas in pre- and post-partum samples of MS patients are consistent with previous data by Ehrlich et al. (34).

The levels of MIF tended to decrease post-partum in MS patients and healthy women. Increased expression of MIF in the blood and cerebrospinal fluid in patients with MS relapses has been reported earlier by us and others (13, 35). MIF is considered to be a pleiotropic cytokine secreted by lymphocytes and macrophages and its most critical functions encompass the regulation of macrophage function, lymphocyte immunity and endocrine functions (36). MIF induces accumulation of encephalitogenic T cells in the CNS thus facilitating the development of EAE (37).

Taken together, upregulation of sTRAIL and sFasL from pregnancy to post-partum situation both in patients with MS and healthy women is the major observation of our study. As these molecules are secreted by immune cells in activation-dependent manner, it is most likely that the quantitative changes detected in this study are a result of T-cell activation occurring during physiological reactivation of mother's immune system after pregnancy (4, 38). An increased risk for relapses in MS after delivery may be associated with changes in immunomodulatory potential of these apoptotic molecules.

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